PRECOCIAL SEXUAL SELECTION

IN

CROTAPHYTUS COLLARIS

By

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Abstract: We investigated the relationships between indicators of fitness and a sexually dichromatic color (hatchling orange bars, HOB) that appears on the side of the body in hatchling male collared lizards (*Crotaphytus collaris*). We found that hatchling females preferred to associate with males bearing brighter HOB and that two measures of HOB were significantly positively related to the probability that hatchling males sire offspring (HOB area) and how many offspring they sire (brightness). Additionally, offspring sired by hatchling males were disproportionately female. Having found the above evidence that HOB are a sexually selected signal, we sought to determine if there was evidence that this signal is maintained honestly. To accomplish this we investigated relationships between measures of HOB and potential indicators of male quality and evidence of costs associated with full HOB expression. HOB area was significantly positively related to hatchling males' immune function, aggression, and testosterone level, but appeared to be negatively related to survival at its highest values. We propose that these findings taken together point to a dual-signal system in *C. collaris* in which some males more fully develop HOB, mate early, then die, while others less fully develop HOB, survive to their yearling season when they develop the typical blues, greens, and yellows of adults and mate then. We further determined the pigment profile of HOB to assess if this signal is a candidate for hypotheses proposing maintenance of honesty via carotenoid trade-off or shared pathways. We found that HOB are overwhelmingly composed of pteridine pigments. Thus, we herein provide evidence for the existence of a pteridine-based precocial sexually selected signal in *C. collaris*.

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CHAPTER I

PRECOCIAL SEXUAL SELECTION IN A REPTILE

Abstract

Sexual dichromatism in animals often is attributed to sexual selection, in which the male (most frequently) is the more colorful sex, using his ornamentation to improve mating success[1]. This phenomenon is well documented in nature across almost all animal lineages from arthropods to primates[1]. In reptiles, sexual dichromatism has been linked to resource holding capacity[2,3], aggression and hierarchical position[4], parasite load[5], and female preference[6,7]. In contrast to the colorful signals of adults, juveniles in most species usually present a dull or substrate-matched coloration, presumably for predator avoidance[8]. A notable exception occurs in the collared lizard, *Crotaphytus collaris*, (and its 8 other congeners), as male (but not female) hatchlings have conspicuous orange bars[9]. This coloration peaks and then fades during their hatchling season and is replaced by adult blue and green coloration. Here we show that more highly ornamented hatchling males are preferred by females, produce more offspring, and produce a higher proportion of female offspring. To our knowledge, this is the first documentation of a precocially

sexually selected signal in any vertebrate species and adds a previously unconsidered dimension to the study of sexually selected signals and the adaptiveness of coloration.

INTRODUCTION

In lizards, conspicuous juvenile coloration is seen only rarely, occurring in some species with tail autotomy or in others as an inter-age-class signal. Conspicuous tails are adaptive in juveniles with the capacity for tail autotomy because tail conspicuousness increases survival by distracting predators from the head and body of the lizard[10,11] and the tail is easily lost, then regenerated[10]. Inter-age-class signals reduce aggression between adults and juveniles by signaling to adults that juveniles are not rivals[12]. In each case the coloration is maintained by natural selection and appears in both sexes.

Crotaphytus collaris presents an interesting alternative to the coloration of most other animals. In a classic example of sexual selection, sexually reproductive individuals manifest strong sexual dichromatism with yearling and older males displaying vivid blue and green body color and yellow throats and heads, while yearling and older females are cryptic and dull (Fig. 1), except that they transiently display orange bars similar to that of hatchling males during the reproductive season[15]. In a novel display, hatchling males develop hatchling orange bars (HOB, Fig. 1) that reach peak intensity then fade prior to their first brumation (hibernation). Herein we use the term "hatchling" to refer to the age class from hatching to first brumation. In spring, lizards emerge and this is assumed to be

when they begin to mate[13]. At this point the yearlings' typical blues, greens, and yellows have replaced the hatchling males' HOB.

Color brightness of yearling and adult males has been associated with female preference and male dominance in some *C. collaris* populations[6]. However, the function and cost of HOB are unknown. Fox and colleagues demonstrated that hatchling males displaying HOB suffered significantly more aggression from hatchling male conspecifics than did males whose HOB were obscured (SFF, Felipe de Jesus Rodríguez, Troy Baird & Andrea Acevedo, in preparation) and bite scarring from these agonistic encounters is frequent in hatchling males from our study site (Extended Data Fig. 1). Furthermore, hatchling males show little to no aggression toward hatchling females, allow their close presence, and even move to maintain body contact with them and display courting-typical behavior. This signaling function in hatchling-hatchling communication contradicts the previous and widespread belief that HOB in *C. collaris* serve as an adult female mimicry signal to reduce aggressive encounters by adult males on hatchlings[14,15]. Moreover, it has been shown that the HOB in *C. collaris* do not deter aggression by adult males[16]. Further, the collared lizard's association with open habitat predicts that any conspicuous color signal should be confined to surfaces easily concealed from potential predators as in inter-age-class signals[12], rather than displayed prominently on the side and back of the body[17]. Such a 'public' signal is expected to experience strong negative natural

selection[17] and is predicted to be maintained instead by sexual selection[18]. Consequently, we hypothesized that hatchling males display a precocious sexually selected signal, with fitness payoffs delivered the next summer as increased number of offspring.

RESULTS

To determine if hatchlings in their first fall season are capable of successful insemination, we tested individuals from each age class and sex for the presence of sperm. We observed sperm in all but one of 28 yearlings and older males in the field (Extended Data Text) and surprisingly, in 58% of 27 hatchling males in the field. These 58% were all sampled at the end of their hatching season just before brumation. All hatchling males larger than 62 mm SVL possessed sperm. These results suggest that late-season hatchling males may be capable of insemination of females prior to their first brumation. For these males to successfully sire offspring, copulated females would need to either store sperm while they overwinter or fertilize eggs and overwinter with arrested embryos. Lizard sperm storage has been documented in at least 12 species with durations from 30 to 540 days (reviewed in[19]) and sperm storage structures have been identified in *C. collaris* females[20].

To determine if hatchling males are siring offspring, we conducted parentage analysis on three years of data collected from a northern Oklahoma population. We successfully assigned a sire to 251 of 303 offspring. Sires were typed as "hatchling" if they were not observed in the population the year after their first brumation and thus,

assumed to be dead (mean total sightings/year = 2324). It is important to note, however, the possibility that these males emerged and mated very early the following year and then died prior to our daily field surveys in spring and summer. Near equal percentages of offspring were assigned to hatchling males (0.35), yearling males (0.36), and males older than two years (0.29). Sex of offspring was significantly related to sire age (χ^2= 3.87, d.f. = 1, $p < 0.05$, Fig. 2). Hatchling males produced more female offspring than did yearlings and males two years and older. Hatchling males produced offspring with females who were of all age classes at the time of mating (H = 0.284, Y = 0.407, A = 0.309) but appeared to be most successful with yearling females.

Upon discovering that males are siring offspring as hatchlings or are successfully securing matings and then dying so early in their yearling season that they are not recorded in the population, we tested for associations between HOB spectrophotometric data (Hue, Saturation, and Brightness), HOB area, and number of offspring sired. We found a significant positive correlation between both brightness ($z_4 = 2.17$, $p = 0.03$; Fig. 3a) and coverage area ($z_4 = -2.02$, $p = 0.04$; Fig. 3b) of hatchling males' HOB and the number of offspring they sired or their probability of siring offspring, respectively. We additionally found that in staged laboratory choice trials, hatchling females (size 59–80 mm SVL, N = 46) significantly preferred to associate with hatchling males with brighter HOB over those with experimentally dulled HOB (linear mixed effect models

comparison: $\chi^2 = 5.09$, d.f. = 1, p = 0.02; Fig. 4a) and hatchling females of 59-70 mm SVL (N = 30, more congruent with the size of hatchling males when they maximally present HOB) showed an even stronger preference for males with brighter HOB (linear mixed effect models comparison: $\chi^2 = 6.03$, d.f. = 1, p = 0.01; Fig. 4b).

DISCUSSION

Collared lizards have evolved a novel system of sexual selection in which hatchlings and adults have evolved completely different signals of male quality within the same species, possibly investing differentially in one strategy or the other. On our study site ~70% of hatchling males die before or immediately after their first brumation. Thus, any successful mating before or immediately following brumation drastically increases posthumous fitness and hatchling males with brighter and more extensive HOB are more successful suitors. If males are mating as hatchlings, they escape the social inhibition imposed by older resident males[21]. Adult males begin brumation earlier in the fall and are not active in the late fall to influence the reproductive behavior of late-season male hatchlings. Over three years, only two adult males were sighted after mid-September and one was encrusted with dirt, apparently having temporarily emerged from brumation, rather than having remained active. Some yearling and adult females (and all hatchlings) remain active into the fall, however. Very early emerging yearlings (March) have sperm-producing testes while adults do not until May[13]. Early emergence could, therefore,

provide mating opportunities in the absence of sperm competition from adult males. Thus, the benefits of an early mating strategy to very young males are clear. While crotaphytid females are known to actively refuse suitors[22], yearlings and older males are larger than females and are capable of forcing copulation (Wiggins pers obs.). Hatchling males do not eclipse hatchling females in size until the end of the hatchling season and are smaller than or equal in size to most yearling and adult females. Thus, when mating with a hatchling male (or perhaps very young yearling), females are potentially able to be more selective in their mates and to cryptically select offspring sires through both pre- and post-copulatory sexual selection ([19]and citations therein). This system could provide females with the opportunity to temporally separate reproductive events, copulating when high quality mates are available and fertilizing/laying eggs when the opportunity for offspring survival is highest.

The fact that hatchling/early yearling males produce more female offspring and our finding, in agreement with previous work on this species[23] that females produce offspring from multiple males, may point to a stronger influence of post-copulatory sexual selection in the form of cryptic female choice and sperm competition than previously thought. Furthermore, while paternity is frequently determined by the relative number of sperm, large numbers of examples exist in which paternity cannot be explained by sperm number, indicating female choice or unmeasured superior sperm

qualities (reviewed in[24]). Female sand lizards respond to more colorful suitors by both biasing their offspring sex ratio toward females and more heavily investing in egg production[25]. In guppies, artificial insemination experiments with equal numbers of sperm from two males unknown to the female demonstrated that colorful males sire a higher proportion of offspring than less colorful males.[26]

Adult females have been found to prefer bright adult males in some *C. collaris* populations[6], and the frequency of display in territorial males (usually 2 years and older) positively correlates with offspring survival[27]. York and Baird suggested that territorial males use display tactics to advertise their fitness to females while subordinate males use stealth[27]. We suggest that there is a third alternative mating strategy in which very young males use HOB, a completely different sexual signal than that used by older males, to enhance mating success prior to or immediately following their first brumation, successfully facilitating posthumous fitness. We call this third alternative precocial sexual selection and suggest that it is a novel twist on classical sexual selection.

METHODS

Field – At our study site, *C. collaris* are active from late April to mid-October. Adults and yearlings emerge from brumation in April and adult males return to brumation in early August followed by adult females and yearling males and females, a few remain active into October. Hatchlings first appear in late July and enter brumation in mid-

October, with all individuals underground by the end of October[28] (Wiggins and Fox pers. observ.). During the active period, a field team composed of 2–7 individuals made daily surveys of the study area 4–6 days a week depending on weather. The study site was a 3.4-ha area on Sooner Lake dam in Pawnee County, Oklahoma (36°27'28.0"N, 96°59'39.8"W), on a substrate consisting of concrete-covered riprap.

Throughout the active seasons of 2014–2017 we captured each lizard using a string or monofilament noose attached to a 4-m telescoping pole. Upon capture, snout-vent length (SVL in mm), mass (g), date, time, and the lizard's identity, location, and sex were recorded. Each lizard received a unique identifier in the form of a toe clip and dorsal non-toxic latex paint dot sequence at first capture. Sex was determined from enlarged post-cloacal scales in males, sexually dimorphic coloration, and morphology. Age was known with certainty when subjects entered the population in the fall as hatchlings. Immigration of lizards from outside the study site (or emigration from the site) was extremely rare. In four years of field work on our site and a site ~ 400 m away, separated by vegetation and connected by a road, only one subject was ever documented as moving between sites.

Sperm Presence Analysis – During the hatchling seasons of 2015–2016, cloacas of males and females from each age group (hatchling, yearling, adult) were swabbed. We captured lizards by noosing, took body measurements (SVL, mass, tail length), everted

the hemipenes (paired copulatory organs) in males or lifted the tail away from the body in females to expose the cloaca. We rubbed a microscope slide across the hemipenes or cloaca and allowed the slide to air dry. We evaluated smears under total magnification of 400x and visually assessed sperm presence or absence[21]. In this species, sperm are naturally green-colored and easily visible without histological staining. Further, the absence of sperm in all females save for two who had just copulated (supplementary data text) serves to confirm our technique.

Color Measurements - In 2014, 2015, and 2016 when male hatchlings reached full orange bar development (SVL = 58–75 mm), we measured their HOB reflectance with a UV-Vis spectrometer (USB 4000, Ocean Optics), deuterium-halogen light source (DH-2000-BAL, Ocean Optics), probe (QR400-7-SR-BX, Ocean Optics) and Spectra Suite software (Ocean Optics) and photographed their lateral body to quantify HOB area. The spectrometer probe was mounted within a probe holder that excluded ambient light and ensured that readings were taken from areas 2 mm in diameter at a constant 7-mm distance from the surface with both illumination and reflectance measurement at a 90° angle to the surface. HOB are approximately 2 mm in width, so reflectance from only HOB was measured. A total of nine readings were taken from each subject, three from each of three bars; these were averaged. Spectral reflectance was measured at 320–700 nm as this represents the broadest range of wavelengths known to be visible to lizards[29].

Lizards were kept under a heat lamp for a minimum of one hour prior to measurement to maintain optimal body temperature. CLR5 (v. 1.05[30]) was used to calculate three standard measures of reflectance for the HOB: hue, saturation, and brightness[31]. Among the indices calculated by CLR5, we selected H_3, S_1R, and B_2 respectively. H_3, also called H_{mid}, uses multiple measurements to calculate hue, reducing the influence of the random fluctuations that occur at each wavelength[31]. S_1R calculates saturation by dividing reflectance in the 605–700 nm wavelength range (orange-red) by total reflectance. B_2, or intensity, calculates mean brightness across all wavelengths and is comparable across studies. Upon completion of spectral readings, photographs of the lateral body of the same males were immediately taken with a ruler for scale. ImageJ software was used to quantify the area of orange relative to total lateral body area.

Parentage - Blood was collected from the toes of all individuals upon toe clipping at first capture in the field and preserved on Whatman FTA classic cards. We extracted DNA from the cards by excising a 3-mm square of blood-saturated card using sterile scissors then followed the GE Healthcare extraction protocol using Chelex® 100 resin. Parentage was determined using eight known microsatellite markers of *C. collaris*[32–35]. Locus Org7 was identified by both CERVUS and MICROCHECKER software as producing null alleles in this population. Analysis retaining Org7 typed at only one allele did not improve parentage assignment. Locus N5 consistently produced

ten or greater amplicons despite extensive clean-up efforts. Therefore, Org7, and N5 were excluded from final parentage analysis.

Polymerase chain reaction amplification was performed as in Supplementary Table 1 using the following formula: 0.5 ul of 1 uM forward and reverse primers, 4 ul nuclease free H_2O, 9 ul TrueAllele, and 1 ul extracted DNA excepting locus Org25, which requires MgCl in addition to that provided in TrueAllele. Org25 was amplified using 0.5 ul of 1 uM forward and reverse primers, 3.25 ul nuclease free H_2O, 9 ul TrueAllele, 0.75 ul 0.25M MgCl and 1 ul extracted DNA. All loci were amplified in an Eppendorf thermocycler. Microsatellite lengths were visualized on an Applied Biosystems 3130 or 3037 genetic analyzer via capillary electrophoresis and allelic size was determined using GeneMapper 4.0 with reference to the ROX size standard. Post PCR product was multiplexed for E48 + O6 and E21 + O21; the remaining loci were not multiplexed. Each analysis reaction contained 9 ul formamide (Hi-Di), 0.5 ul GeneScan 400HD Rox size standard, and 1 ul post PCR product for each locus in the multiplex reaction. We determined null allele frequencies in MICROCHECKER and CERVUS and made parentage assignments using LOD score and delta in CERVUS[36].

Female choice – We captured hatchlings for female choice trials from our field site and nearby populations and transported them to the laboratory for staged trials. Males used in paired choice trials were size matched within 2 mm SVL and did not originate

from nearby the capture location of the tested female. At random, one male's HOB were "dulled" using watered down non-toxic light beige latex paint (Anita's All Purpose Acrylic–11129 Café au Lait) that spectrally matched the background non-HOB coloration of hatchlings (Extended Data Fig. 2) while the other male's HOB were left untouched. These males were subsequently named the "dull male" and "bright male." One male of each type (dull and bright) was placed in each of two 38-l glass aquaria arranged side-by-side and a female was placed in a 38-l aquarium set perpendicularly to allow her clear visibility of each male. A visual barrier was placed between the males at all times and between the female and males at the time of placement. After a ten-minute acclimation period, the barrier between the female and males was lifted. The time the female spent on the side of her tank immediately in front of each male's tank was recorded. Trials ran a total of 30 minutes. Trials in which the female did not move were excluded from analysis.

We evaluated the effectiveness of dulling males' HOB by painting 10 swatches of HOB spectrally matched orange paint (Extended Data Fig. 2) on a white sheet of paper and taking the spectral measurements of each swatch at two locations as described above. We subsequently painted a watered-down thin layer of the light beige colored paint just as on the hatchling males and took spectral measurements in the same locations as before.

We found brightness to be significantly lower in the beige-painted measurements (paired t-test, $t_{19} = 3.7$, $p < 0.01$).

Statistical Analyses - Data were analyzed in R version 3.4.2[37]. Correlations between HOB spectrophotometric variables and HOB area and between HOB color variables and SVL were tested in an ordinary least squares linear model. All spectrophotometric variables were significantly correlated with each other ($p < 0.001$) and with HOB area ($p < 0.05$) except brightness, which was not correlated with area ($p = 0.17$). HOB saturation and brightness were both significantly correlated with SVL ($p < 0.03$). Thus, each spectrophotometric color variable was analyzed as a separate predictor and residuals were extracted for saturation and brightness for downstream analyses. We analyzed correlations between sperm presence and snout vent length in a generalized linear model with binomial errors. All individuals 62 mm SVL and larger possessed sperm and all individuals 61 mm SVL and smaller did not, which led to a fitted probability of 1. Deviations from expected offspring sex ratios for hatchling vs. yearling and adult (combined) sires were evaluated using Chi-squared tests, and correlations between number of offspring sired and sire HOB color and HOB area were evaluated using a zero-inflated model with Poisson errors[38] as these were zero-inflated count data. To analyze our female choice data, we first evaluated time spent with each male ("bright" and "dull") for normality using the Shapiro-Wilks test. Upon finding this variable to be

normally distributed, we employed a linear mixed effects model. In our model, male color "bright" or "dull," was set as the fixed effect, female ID as the random effect, and time spent with each male as the response variable[39]. The explanatory power of our model was evaluated by a likelihood ratio test. Specifically, we compared our model employing male color as a fixed factor to a null model that did not include male color as a fixed factor[40]. The resulting test statistic is Chi-squared distributed (Wilks' theorem[40]).

EXTENDED DATA TEXT

We did not find sperm in the hemipenes swab of one yearling male. This was one of the first animals to be swabbed and probably relates to our initially inadequate techniques while we were perfecting the method rather than true absence of sperm.

In 2016 a hatchling male and yearling female from our study site were captured and transported to the lab. They were enclosed in a neutral arena bisected by a removable divider, allowed ten minutes to acclimate, then allowed 30 minutes to interact. JMW observed the interaction. The male and female showed no aggression, but rather produced courting-typical behavior (head bobbing, nose touching without aggression–lateral displays or lateral displays with dewlap extensions). After approximately 15 minutes the yearling female permitted the hatchling male to mount. The male did not bite the back of the female's neck as is typical in mating between older collared lizards and the subjects were positioned such that the cloacas were not visible. The male appeared to attempt to

move his pelvis under hers. The typical female tail-raise was not observed. After approximately two minutes the female moved slowly forward and the male appeared to be dragged by his pelvis with her. After this interaction, the male and female appeared to ignore each other for the remaining observation time. Swabs of the cloaca and hemipenes revealed sperm on the male's hemipenes and a single sperm was recovered from the female's cloaca.

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Table S1: Microsatellite loci used in parentage analysis. Locus names are as given in Hutchinson (2004)

Name	Length	Repeats	Annealing temp
Enr3	101-116	3	58
Org21	126-160	2	62-50 TD*
Enr48	102-138	2	48
Org6	138-150	2	46
Enr21	99-114	3	58
Org26	99-139	2	52
Org24	144-204	4	50
Org25	109-154	3	49

*TD = touch down PCR

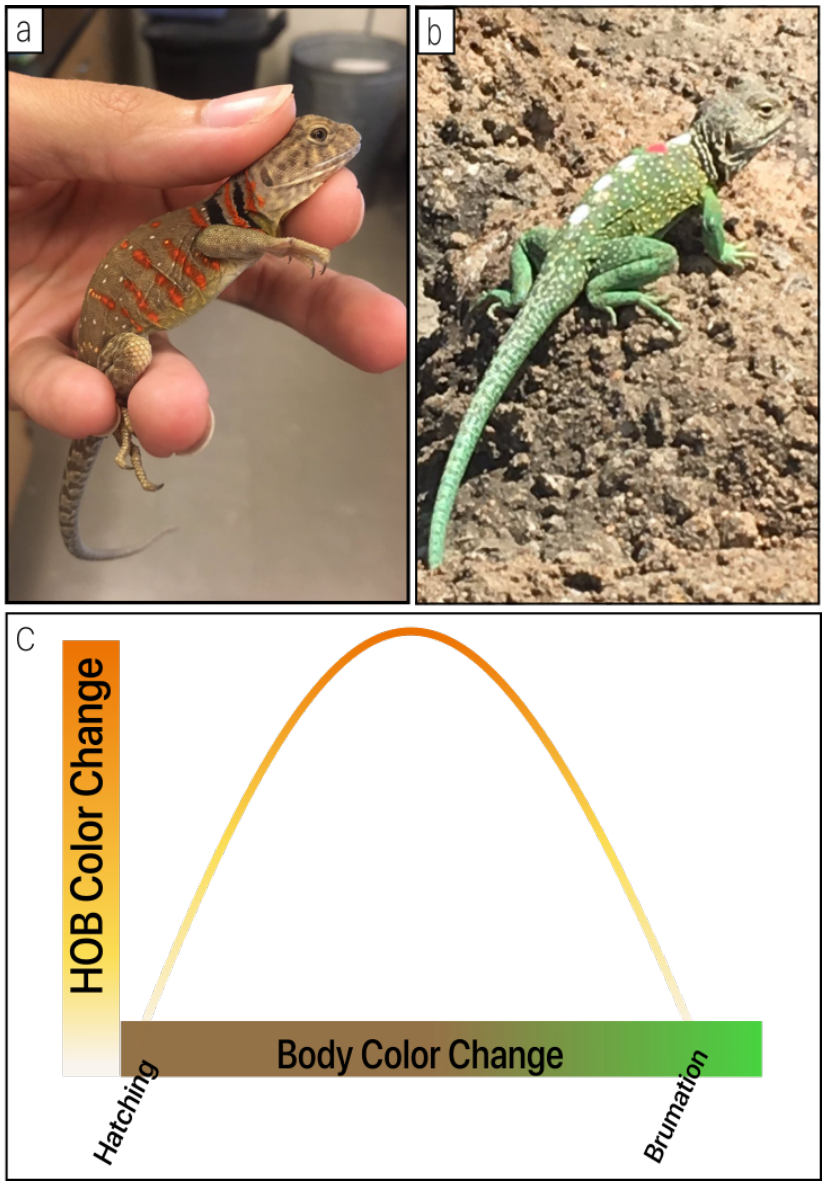


Figure 1 | Hatchling and adult male coloration and developmental timing of hatchling orange bars (HOB). **a**, Hatchling *C. collaris* male displaying fully developed sexually dichromatic HOB. **b,** Adult *C. collaris* male displaying typical sexually dichromatic full body blues and greens. **c,** Representation of the approximate timing of HOB development and loss relative to hatching and first brumation.

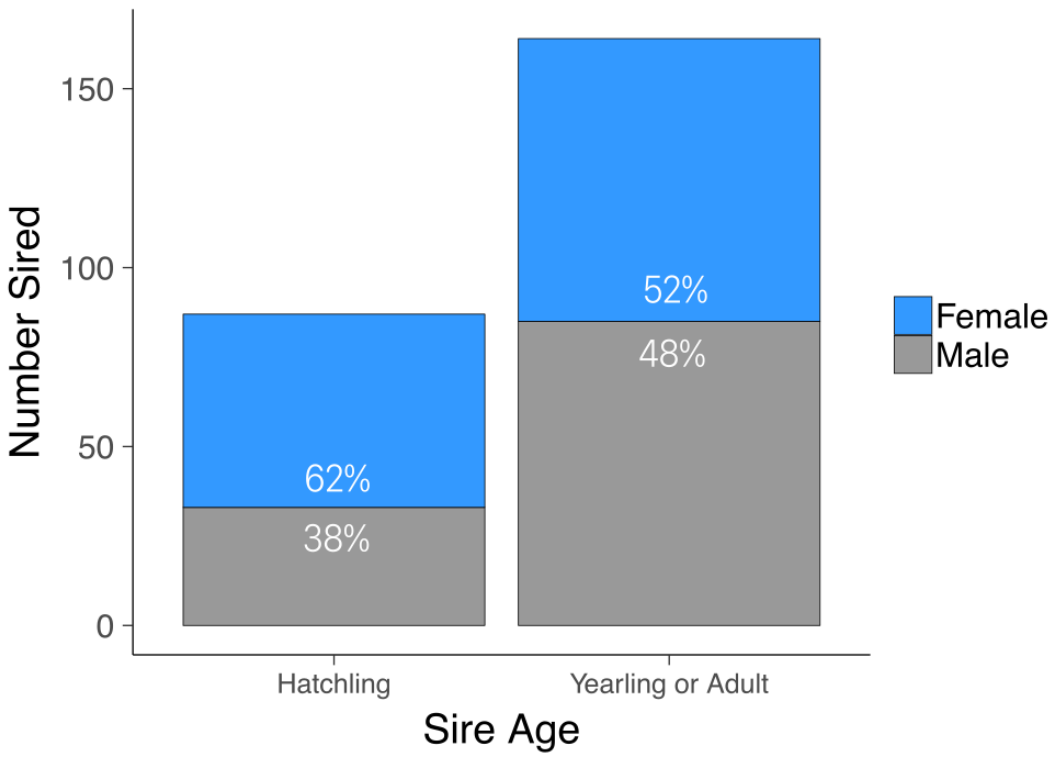


Figure 2 | Proportion of female and male offspring sired by hatchling or yearling and adult males. Hatchling *C. collaris* males sire a significantly higher proportion of female offspring than do their yearling and adult counterparts (χ^2= 3.87, d.f. = 1, $p < 0.05$).

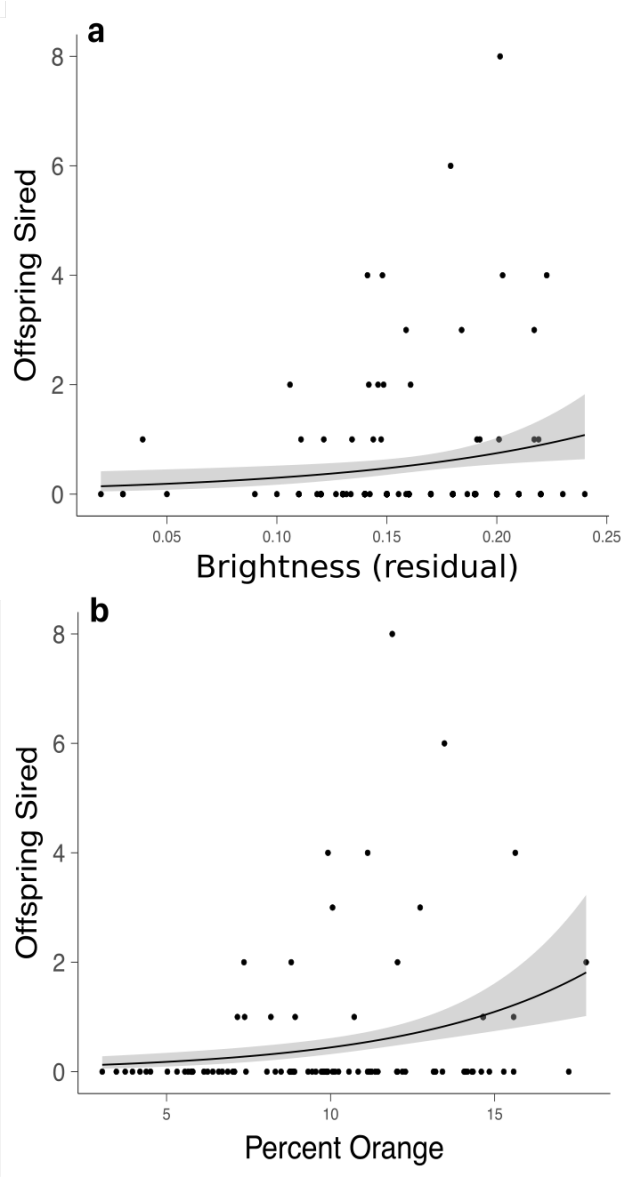


Figure 3 | Relationships between HOB and number of offspring sired. a, HOB brightness (residual of regression with SVL) significantly related to number of offspring sired in the truncated Poisson portion of the zero-inflated model, indicating that as HOB becomes brighter males are more likely to sire larger numbers of offspring. **b,** HOB area significantly related to number of offspring sired in the binomial portion of the zero-inflated model, indicating that males with more HOB coverage are more likely to sire offspring. Grey shading represents 95% confidence interval.

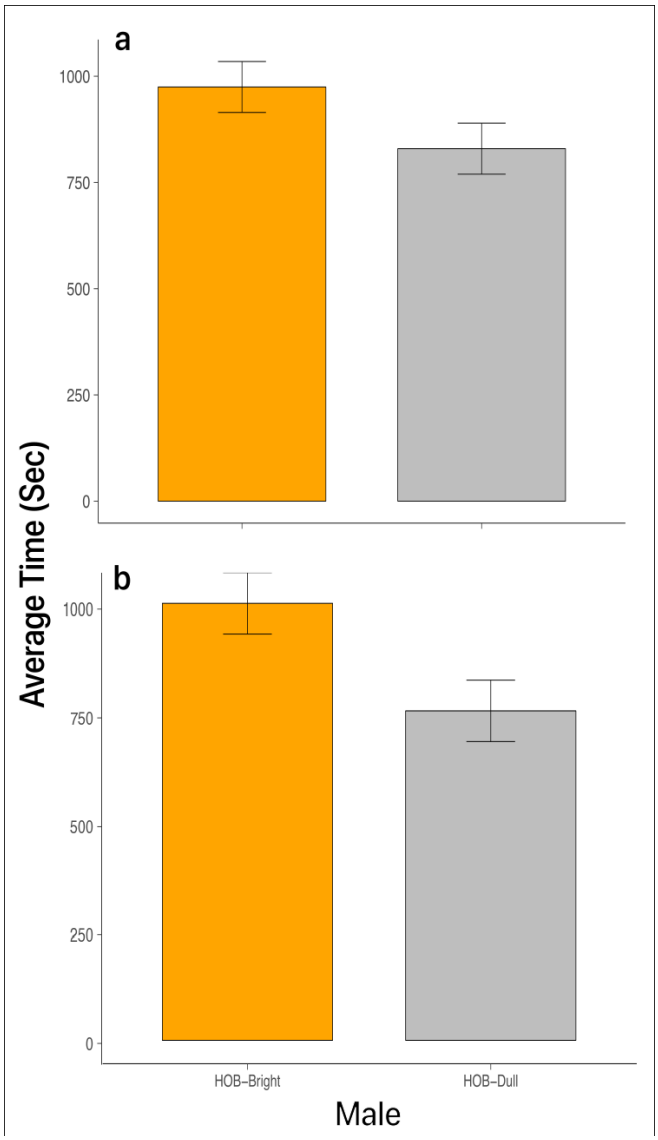
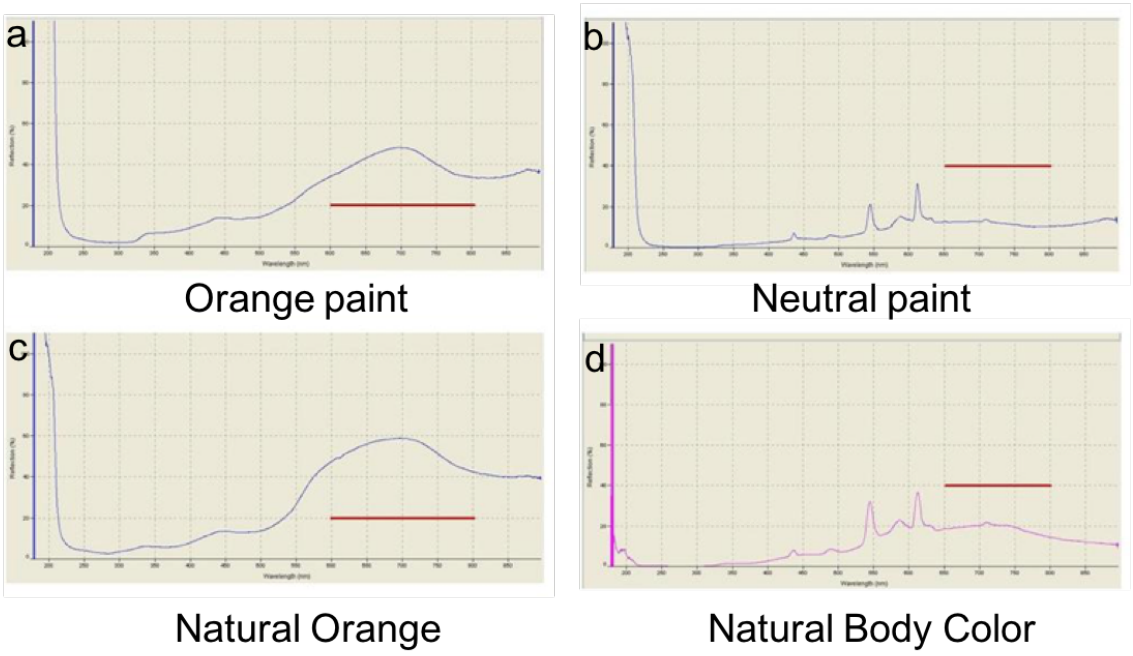


Figure 4 | Hatchling female preference for hatchling males with brighter HOB. Mean time ± SE a, Females of 59--80 mm SVL spent significantly less time with experimentally dulled males (-185.13 sec. ± 80.87) than with naturally bright males ($\chi^2 = 5.09$, d.f. = 1, p = 0.02). **b,** A subset of females (SVL 59--70 mm SVL) whose size matches that of hatchling males at peak HOB development preferred the brighter male even more strongly (-252.97 sec. ± 100.54 for the dull male) than did females in the full range of sizes ($\chi^2 = 6.03$, d.f. = 1, p = 0.01).

Extended Data Figure 1 | Bite scarring (matching the jaw size of a hatchling *C. collaris* individual) on a hatchling male bearing HOB.



Extended Data Figure 2 | Spectral reflectance curves of **a,** the orange paint used to assess efficacy of **b,** the neutral paint used to dull hatchling males' HOB in female choice trials as compared to **c,** natural HOB, and **d,** natural hatchling male (non-HOB) body color.

CHAPTER II

INTEGRATED MEASURES OF MALE QUALITY IN A PRECOCIAL SEXUALLY SELECTED SIGNAL

Abstract - Hatchling male collared lizards (*Crotaphytus collaris*) display a conspicuous orange signal–hatchling orange bars (HOB)–that is entirely different than adult male coloration. Measures of HOB are positively related to female preference, number of offspring sired, and probability of siring offspring, indicating this is a precocial sexually selected signal. Here we assessed measures of signal honesty by testing relationships of sex steroids, immune function, aggression, activity and display rates, and growth with HOB. We found that HOB area was significantly related positively to testosterone and aggression, tended to be positively related to immune function, but tended to relate negatively to survival. Conversely, HOB brightness was significantly related negatively to androgens and tended to be positively related to survival. HOB hue and saturation appeared to be under stabilizing selection. We further determined that HOB are overwhelmingly composed of pteridine pigments. We propose that our findings regarding HOB area taken together with this measure's positive relationship with male fitness point

to a dual signal system in this species in which hatchling males with well-developed HOB mate early and die (precocial sexual selection) while others with less well-developed HOB survive to become yearlings, develop adult typical blues, greens, and yellows, and subsequently mate (classic sexual selection).

INTRODUCTION

Sexually Selected Signals – One of the primary tenets of sexual selection theory is that sexually selected signals (SSSs) must be maintained honestly–the signal accurately transmits information about the signaler's quality–to be evolutionarily stable (Zahavi 1975, 1977; Hamilton and Zuk 1982; Kodric-Brown and Brown 1984; Enquist 1985; Pomiankowskl 1987; Grafen 1990; Folstad and Karter 1992). Color can be a reliable signal of quality in male lizards signaling to females (Thompson and Moore 1991; Baird et al. 1997; Kwiatkowski and Sullivan 2002; Whiting et al. 2003; Cook et al. 2013) as well as to rival males (Baird et al. 2013), even eclipsing the importance of body size (Calsbeek and Sinervo 2002). The mechanism by which honesty is maintained is of particular interest, as it would serve signalers to display signals that artificially inflate their perceived quality (to "cheat"). Zahavi (1975, 1977) proposed that sexually selected traits confer handicaps on the possessor and that only the highest quality individuals can afford to be handicapped and still survive (Handicap Principle). Meanwhile, Hamilton and Zuk (1982) demonstrated that blood parasite load correlated with brightness and song

in several bird species and proposed that this demonstrated inherently "good genes" (as genes for parasite resistance will always remain heritable) possessed by those males capable of resisting parasites, resulting in an unbluffable display. Folstad and Karter (1992) built on the handicap and good genes hypotheses by linking decreased immune function with the primary androgenic hormone, testosterone, formulating the immunocompetence handicap hypothesis. This hypothesis states that high levels of testosterone both increase sexually selected traits and decrease immune function, thus increasing parasite burden. The above hypotheses have met with both criticism and support (Pomiankowski 1987; Grafen 1990; Számadó 2011). Kodric-Brown and Brown (1984) proposed that honest advertising need not be expressed as a handicap to survival, and asserted that sexual selection favors traits whose expression is correlated with genetic fitness and that the expression of these traits is based on a tradeoff of resources, concluding that only those individuals who can "afford" highly expressed SSSs are the most genetically fit. Maynard Smith and Harper (1995) termed this an "index" signal, stating that signal intensity is correlated with individual quality through some shared physiological process. Recently, mitochondrial function (Hill and Johnson 2012; Johnson and Hill 2013; Hill 2014) has been proposed as a shared pathway for red/orange coloration produced by carotenoids (Vitamin-A redox hypothesis). It is important to note that the mechanism by which signal honesty is maintained likely varies based on life history strategy and lack of support for one hypothesis in any system does not preclude

its function in another (Morehouse 2014). Additionally, it is entirely plausible for multiple hypotheses together to explain the maintenance of signal honesty (Weaver et al. 2017).

Red/Orange coloration – Unlike in birds and mammals, where pigments are deposited into dead keratinized cells (feathers or hair, respectively; McLean et al. 2017), reptiles produce color via three layers of chromatophores in the dermis of the integument (Grether et al. 2004; Olsson et al. 2013). The most superficial, xanthophore layer, houses carotenoid and/or pteridine pigments and is responsible for producing yellow-red by absorbing short (violet-blue) and reflecting long (yellow-red) wavelengths (Grether et al. 2004; Olsson et al. 2013). The production of red/orange coloration requires the dynamic production/sequestration of carotenoids and/or pteridines. Carotenoids must be obtained from an animal's diet and have been proposed as a limiting resource. Further, specific types of carotenoids can serve as antioxidants as well as pigments for ornamentation (Lozano 1994; Møller et al. 2000). Thus, color produced by carotenoid pigments is a subject for hypotheses invoking costs or handicaps to maintain honesty (resource trade off and immune function explanations) along with the Vitamin A-redox (shared pathway/index) hypothesis. Pteridines, conversely, are synthesized endogenously from guanosine triphosphate (Ziegler 2003; Braasch et al. 2007) and are the most significant pigments contributing to red/orange color in fish, amphibians, and non-avian reptiles

(Macedonia et al. 2000; Bagnara and Matsumoto 2006; Steffen and Mcgraw 2007; Weiss et al. 2012; Cuervo et al. 2016; Merkling et al. 2016; McLean et al. 2017).Yet, the specific connections between pteridine-based color and maintenance of honesty are little explored. The differential use of carotenoids and/or pteridines to produce a red/orange color leads to different evolutionary and physiological constraints on the signal and on the methods by which their stability is maintained (Olsson et al. 2013).

Crotaphytus collaris, the Eastern collared lizard, presents an interesting alternative to other reptilian color expression. Adult and yearling individuals manifest typical sexual dichromatism, with the male displaying vivid blues, greens, and yellows while the female remains dull in color. But immediately following hatching, hatchlings of both sexes vary in coloration with some individuals possessing orange bars along their lateral body and other individuals being dull grey-brown and chromatically unremarkable. Orange coloration fades quickly (within a week) after hatching in *all* individuals. However, in a novel display, as the hatchling males grow, they soon develop conspicuous orange bars running vertically along their sides. These hatchling orange bars (HOB) reach peak intensity during the hatchling's first season and then begin to fade to be replaced just before brumation (hibernation) by the blue and green dorsal coloration and yellow throats characteristic of sexually mature *C. collaris* males. Some males,

especially small ones, maintain the HOB into the beginning of their yearling season, but the color soon fades entirely.

Remarkably, hatchling females are uniformly dull brown at this stage (see below). Conversely, while yearling and adult males maintain a static, green-blue body color, females during the reproductive season develop temporary conspicuous red-orange bars running vertically down their sides (McGuire 1996). Much work has been undertaken to determine the ecological function of the female orange bars (Cooper and Ferguson 1972; McGuire 1996; Baird 2004). It was previously thought that the female orange bars served a deterrent purpose, informing potential mates that the female was gravid (Cooper and Ferguson 1972). Recent work has demonstrated that the orange bars are much more likely to advertise reproductive receptivity (Baird 2004). In *Crotaphytus*' sister genus, *Gambelia*, similar female coloration has been hypothesized to deter attempted copulations (Montanucci 1965; Tollestrup 1983), but as in *Crotaphytus,* recent work has pointed to a stimulatory purpose (Germano and Williams 2007).

While years of work have focused on the adults of this species, until recently little inquiry has been undertaken to ascertain the function and cost of hatchling orange bars (HOB). We know that hatchling males are more aggressive to other hatchling males that display HOB; if a conspicuously colored male's HOB are covered with body-matched paint, the aggression of his counterparts decreases markedly (Fox et al. 2011).

Furthermore, hatchling males show little to no aggression toward hatchling females, who have no HOB. This signaling function in hatchling-hatchling communication contradicts the previous and widespread belief that HOB served as an adult female mimicry signal to reduce aggressive encounters by adult males on hatchlings (Fitch 1967; Cooper and Ferguson 1972; Cooper and Greenberg 1992; Carpenter 1995; McGuire 1996). Moreover, it has been shown that the HOB in *C. collaris* do not deter aggression by adult males (Husak et al. 2004). Additionally, adult male *C. collaris* are less aggressive toward adjacent territory holders than to novel males, adhering to the "Dear Enemy" effect (Husak and Fox 2003). Adult males can recognize neighbors even outside the context of their territories (Husak and Fox 2003). Given that males recognize and respond differently to adjacent territory holders (even out of context), it is plausible that hatchlings will recognize and remember other hatchlings, and the social relationships established with them as hatchlings, into later life. Therefore, HOB can serve as an honest signal to rival males and possible mates even after their hatching year and after they have lost their HOB.

Further, the collared lizard's preference for and near perpetual occupation of open habitat predict that any conspicuous color should be confined to surfaces easily concealed from potential predators as in inter-age-class signals (Fresnillo et al. 2015b), rather than displayed prominently on the side of the body (Stuart-Fox and Ord 2004). Such a 'public'

signal is expected to experience strong natural selection (Stuart-Fox and Ord 2004). However, in adults, conspicuous sexual dichromatism frequently results from sexual selection (Andersson 1994; Owens and Hartley 1998; Figuerola and Green 2000; Dunn et al. 2001). Fox and colleagues (2011) demonstrated that males displaying HOB suffered significantly more aggression from conspecifics than did males whose HOB were covered and obvious bite scarring from these encounters is frequent in hatchling males from our study site (J.Wiggins, pers.observ.; Chap 1 Fig. 3). Furthermore, conspicuously painted yearling *C. collaris* lose foraging opportunities (Baird 2008). Given that HOB do not serve as an inter-age-class signal, appear prominently on the lateral body rather than a less critical body part (e.g., the tail: Bateman and Fleming 2009; Fresnillo et al. 2015a), are sexually dichromatic, increase aggression and injury in hatchling males, and likely decrease foraging opportunities, we hypothesized that the HOB are a precocious sexually selected signal. We have previously provided evidence for exactly that, demonstrating the link between HOB and hatchling male reproductive success and hatchling female preference for HOB (Chapter 1).

Based on the finding that HOB are a sexually selected signal, we sought to determine if they are an honest signal. We tested whether HOB intensity is correlated with: 1) immune capacity as predicted by the Hamilton & Zuk "good genes" hypothesis and the immunocompetence handicap hypothesis, 2) sex steroid production as predicted

by the immunocompetence handicap hypothesis, 3) aggression, movement, and growth as indicators of overall condition and measures of costs/benefits of HOB, and 4) survival. We additionally sought to determine the pigment composition (pteridine or carotenoid) of HOB to determine if this signal is a candidate for the Vitamin A-redox shared pathway hypothesis.

METHODS

Field - *C. collaris* is active from April to October. Adults and yearlings emerge from brumation in April and begin to return to brumation in August (especially adult males). Hatchlings begin emerging in late July and begin brumation in mid-October, with all individuals in brumation by October's end (Trauth et al. 2004, J. Wiggins and S. Fox, pers. observ.). During the active season, a field team of 2–7 members made daily surveys of the study site (SL1) 4–6 days a week depending on weather. The SL1 study site is a 3.4-ha area on Sooner Lake dam in Pawnee County, Oklahoma, on a substrate consisting of concrete-covered riprap. Painted rocks serving as numbered flags are spaced approximately 11 m apart such that several can be seen from any given location, and accurate locations can be determined for each lizard sighting by visual estimation of relative distance between two adjacent flags and relative height along the width of the dam and the road on top in six categories of distance from the water.

Throughout the course of the active season of 2014–2017, we captured all lizards on the study site using a string or monofilament noose attached to a 4-m telescoping pole. Upon capture, snout-vent length (SVL in mm), mass (g), date, time, and the lizard's identity, location, and sex were recorded. Each lizard received a unique identifier in the form of a toe clip and dorsal non-toxic latex paint dot sequence at first capture. Sex was determined from enlarged post-cloacal scales in males, sexually dimorphic coloration, and morphology. Age was known with certainty when subjects entered the population in the fall as hatchlings. Immigration of lizards from outside the study site (or emigration from the site) was extremely rare. In four years of field work on our site and a site ~ 400 m away, separated by vegetation and connected by a road, only one subject was ever documented as moving between sites.

Over the course of the active season, 2000+ sightings were recorded per year. These data provide information on space use and social interactions. Observed consorting, conflict, and copulations were recorded, providing a record of social associations and potential mate pairing. Each individual was located multiple times each season. Additionally, data collected on the same site from 2011–2013, using the same techniques but with fewer observations per year (~500), were combined with data from 2014–2017 to quantify survival.

Color Measurements – We assigned subjective categories of HOB intensity in the field ranging from 0 (complete absence of orange) to 3 (most intense orange). When male hatchlings reached most intense HOB according to visual field assessment (SVL = 58–81 mm, Acevedo Crosby, 2015), they were transported to the laboratory and their HOB color was measured with a UV-Vis spectrometer (USB 4000, Ocean Optics), deuterium-halogen light source (DH-2000-BAL, Ocean Optics), probe (QR400-7-SR-BX, Ocean Optics) and Spectra Suite software (Ocean Optics). The spectrometer probe was mounted within a probe holder that excluded ambient light and ensured readings were taken from areas 2 mm in diameter at a constant 7-mm distance from the surface with both illumination and reflectance measurement at a 90° angle to the surface. Reflectance values were recorded relative to white (WS-1-SL diffuse reflectance standard, Labsphere) and black (SpyderCube) standards (Andersson and Prager 2006). The probe was recalibrated after every three lizards assessed.

HOB are approximately 2 mm in width, so color only of HOB was measured. Spectral reflectance was measured at 320–700 nm (scans to average = 20, boxcar width = 10) as this represents the broadest range of wavelengths known to be visible to lizards (Loew et al. 2002). Lizards were kept under a heat lamp for a minimum of one hour prior to measurement to ensure optimal body temperature. CLR5 (v. 1.05, Montgomerie 2008) was used to calculate three standard measures of reflectance for the HOB (as described in

Chapter 1; Montgomerie 2006). Upon completion of spectral readings, photographs of both sides of the body of the same males were immediately taken in the laboratory and photographed with a ruler for scale (all photographs were taken by JMW; lizards were held in the same position). Image-J software was used to quantify the HOB area relative to total lateral body area. HOB area was calculated as the sum of the HOB area from each side (mm^2) divided by the total lateral body area (mm^2) of each side summed. Lateral body area was defined horizontally from just behind the ear to the tail base and vertically from the ventral border between brown body and white belly dorsally to the spine. We assessed the accuracy of our subjective HOB categories by analyzing relationships between these and measured color variables.

Bacteria Killing Analysis **-** In 2014, 15 hatchling males (SVL 66–81 mm) were assessed for immune function with a bacteria killing assay (BKA, Millet et al. 2007). Approximately fifty microliters of whole blood were extracted via post-orbital sinus puncture with a sterile hematocrit microcapillary tube after anesthesia with inhaled isoflurane. Ten microliters were retained for BKA, and the remainder was used for radioimmunoassay (see below). Blood was immediately placed in an ice bath and transported to a sterile hood. Whole blood was combined with an *E. coli* (ATCC #8739) media dilution (CO2 independent media; Gibco, Invitrogen) that yielded 100–250 bacteria colonies on test plates incubated at 37°C for 30 minutes. All samples were run in

duplicate. Fifty microliters of the incubated mixture were plated on tryptic soy agar plates that had previously been prepared and spread with sterile glass beads for 1 minute. Two control plates with no blood sample were prepared in duplicate before and after experimental plates (total = 4) and incubated with each experimental run. Plates were allowed to dry and then incubated for 12 hours at 37^{o} C. After incubation, we counted individual colonies on each plate. Experimental duplicates and control quadruplicates were averaged. To determine bacteria killing capacity, the average colony count for each lizard was divided by the control average. The resulting value was subtracted from one to attain the proportion of bacteria killed.

Wound Healing Analysis - As a second integrated measure of immune function (French et al. 2006), 11 males in 2015 not used in other trials received a 3-mm cutaneous wound over the pelvis slightly lateral to the vertebral column on the left side via sterile biopsy puncture. Wounded males were then returned to their exact site of capture after two days post-op recovery in the laboratory. Males were recaptured at each subsequent sighting post-wounding and the wound was photographed in the same position and with a ruler for standardization. Photographs were analyzed for decreasing wound diameter with Image-J software with the goal of calculating time to healing. Blood was not drawn from wounded individuals for sex steroid quantification.

Sex Steroid Quantification **–** Approximately 50 µl of whole blood were drawn from all hatchling males that reached maximal HOB development (SVL 58–81; Acevedo Crosby 2015) via the same method as described above for BKA. Blood was separated by centrifuge (5 min at 6000 rpm), and plasma was extracted from blood samples and frozen at -20°C within eight hours of bleeding. Prior to analysis, plasma samples were thawed and volume used for the assay was recorded to the nearest µl using a Hamilton syringe, after which they were mixed with 0.5 ml dH_2O to provide sufficient volume for steroid extraction. Plasma concentrations of testosterone (T), 5α-dihydrotestosterone (DHT), 17ß-estradiol (E2), and progesterone (P) were measured by standard radioimmunoassay (RIA) techniques following extraction and chromatographic separation (Wingfield and Farner 1975; Wack et al. 2008). Samples were equilibrated overnight at 4°C with 1000 cpm each of tritiated T, DHT, E2, and P (Cat. Nos. T: NET-370, DHT: NET-453, E2: NET-317, P: NET-381) for individual recovery determinations. Plasma samples were then extracted twice with diethyl ether, dried in a 37°C water bath under nitrogen gas, and reconstituted in 500 µl of 10% ethyl acetate in isooctane.

To isolate T, DHT, E2, and P, all samples were transferred to columns of diatomaceous earth (Celite, Sigma, St. Louis, MO) with a Celite:ethylene glycol:propylene glycol upper phase (6:1.5:1 m:v:v) and a Celite:ddH2O (3:1 m:v) lower phase for chromatographic separation. Neutral lipids were eluted with 2.0 ml isooctane

and discarded. P, DHT, T, and E2 were eluted with (P) 2%, (DHT) 10%, (T) 20%, and (E2) 40% ethyl acetate in isooctane, respectively, and collected in test tubes. After this, samples were dried in a 37°C water bath under nitrogen gas, resuspended in phosphate-buffered saline, and maintained overnight at 4°C. Competitive binding RIAs were performed using the respective tritiated steroid tracer (above) and antisera (Cat. nos. T & DHT: T-3003 (Fitzgerald Industries (Formally Wein Laboratories: Concord, MA), E2: 7010-2650 (Biogenesis, Kingston, NH), P: P-5289 (Sigma, St Louis, MS). We ran standard curves in duplicate and samples singly to maximize detectability. To estimate assay precision, four aliquots from a standard pool, treated the same as above samples, were run in each assay. Intra-assay coefficients of variation (CVs) for T, DHT, E2, and P were 11%, 23%, 43%, and 18%.

Growth – Growth per day of SVL (mm), tail length (mm), and mass (g), were calculated for 68 hatchling males by subtracting the first measurement taken from the last and dividing by the number of days between (e.g., (SVL at last measurement - SVL at first measurement)/number of days between measurements), regressing this value against the starting measurement and extracting the residual as an estimate of daily growth.

Aggression – Hatchlings were paired with a size-matched (within 3 mm SVL) novel, stimulus male. Stimulus males' HOB were slightly dulled using diluted non-toxic body-matched paint (Anita's All Purpose Acrylic–11129 Café au Lait, Chap 1 Extended Data

Fig. 2) to prevent subject males' avoiding interaction due to potential for perceived opponents' dominance. However, dulled HOB were visible in stimulus males. After a minimum of an hour under heat lamps to ensure an active body temperature, each pair was placed in a divided neutral arena in a novel room. Immediately after placing the lizards in the chamber, the observer moved behind a blind (black curtain with viewing slit hung from the ceiling). The arena was bisected by an opaque panel to allow the stimulus and the subject lizards to acclimate to the arena before first seeing each other. The lizards were given a 10-minute acclimation period, after which the bisecting panel was lifted remotely via a pulley. Time to first aggressive act was recorded, after which each interaction was allowed to run for 10 minutes. Tallies of 10 different aggressive acts over the course of the 10-minute interaction period were made and the total of weighted aggressive acts (+1: approach; +2: throat display, lateral throat display, headbob, pushup, circle, gape, superimposition; +3: attack, bite, fight) less weighted submissive acts (-1: retreat, flee) was calculated.

Focal observations – In 2015 and 2016, we carried out between one and four 20-minute focal observations on 27 hatchling males. We attempted to carry out these focal observations as close to peak HOB as possible. Focals were conducted as follows: A hatchling male was identified by his unique paint sequence through binoculars. At the time of siting, the date, time, and temperature (recorded from Weather Underground)

were recorded along with the lizard's ID and location. The observer subsequently retreated to a minimum distance of 20 m and started a timer. Focals were dictated into an Olympus digital voice recorder (model VN-6200PC or VN-701PC) or the voice record function on the observer's cellular phone. During focal observations, total displays (as in Aggression above), number of movements, total distance moved, and intrasexual and intersexual interactions were recorded.

Orange/Red Pigment Analysis – One hatchling male was euthanized via inhaled isoflurane and immediately frozen intact. Subsequently, multiple (10 +) ~ 5 mm^2 sections of HOB-bearing skin were removed via sterile scissors under a dissecting microscope. Skin samples were divided into two microcapillary tubes containing 1500 ul of methanol. Pigments were extracted from the skin as in McLean et al. (2017). Briefly, a sequential carotenoid and pteridine extraction was used. Samples were dried, weighed and homogenized in methanol:ethylacetate using a TissueLyser II system (and 3-mm tungsten-carbide beads, Qiagen, Hilden, Germany). The resulting carotenoid extract was collected following each of two rounds of extraction and centrifugation. Pteridines were extracted from the remaining tissue pellet using 2% ammonium hydroxide. Carotenoids and pteridines were quantified in separate LC-MS analyses on an Agilent 6490 triple quadrupole MS system with a Jet Stream electrospray ionization source coupled to an Agilent 1290 series LC system (Agilent Technologies Inc, Santa Clara, CA). Data

analysis was performed using the Agilent MassHunter Workstation Software (version B.07.00). Pure forms of each pigment were purchased (excepting drosopterin, which we extracted) and analyzed to provide standard peaks. All sample peak assignments were matched against standard peaks and confirmed with a qualifier ion, which also aligns with the peak and retention time.

Statistics – Statistical analyses were performed in R 3.4.2 (R Core Team 2017). Data collected from 2014–16 included sex steroids, spectral HOB color variables, HOB area, growth, aggression, and bacteria killing ability (BKA was conducted only in 2014). Data collected from 2011–17 included HOB spectral color variables and survival. HOB area was available only for 2014–16, thus survival for this variable was assessed using the smaller data set.

For all variables, we checked for normality using the Shapiro-Wilks test. Sex steroid measurements were log transformed to achieve normality. We separately regressed HOB color variables, sex steroids, and aggression on SVL in OLS linear models. For those variables significantly related to SVL (E2, Aggression, B2, S1R, H3; only in the 2014–16 data set), we extracted residual values for use in subsequent analyses. We assessed correlations between variables using Pearson's coefficients (Harrell and Dupont 2018) and found significant correlations between all spectral HOB color variables ($p < 0.001$) and between HOB area and hue ($p = 0.02$). Nevertheless, we used all variables in

analyses, but not multivariate analyses to avoid multicollinearity. One aggression score, which was more than four standard deviations greater than the mean, was excluded from final analysis. Regressions between variables in the 2014–16 data set (sex steroids, spectral HOB color variables, HOB area, growth, aggression, and BKA) were subsequently conducted pair-wise in OLS linear models.

For survival analyses (2011–2017 data), we assessed relationships between HOB color variables and survival to yearling and survival to adult separately in generalized linear models with binomial errors (logistic regression, 0 = did not survive, 1 = did survive). We tested for directional (linear) and stabilizing (quadratic) selection (Janzen and Stern 1998; Wood 2017). HOB area was collected only from 2014–2017. Of the 86 males from whom HOB area data were collected, only two survived to be adults. Thus, survival to adult as a function of HOB area could not be reliably evaluated. Noting that no individual with the highest 11 values of HOB area survived to yearling, we employed 1000 iterations of random sampling of survival values (0,1) from our data set. From this we calculated the probability of 100% of a set of 11 individuals not surviving to yearling.

For focal observations, relationships between response variables (number of movements, number of displays, total distance moved, and social interactions) and predictor variables (sex steroids, spectral HOB color variables, HOB area, temperature, time, and elevation) were assessed using a generalized linear mixed model with lizard ID

as a random variable. Social interactions were evaluated with binomial errors (1 = interaction, 0 = no interaction), while movement and display variables were assessed with negative binomial errors due to overdispersion. Temperature was significantly related to number of movements and was included as a co-variable in all subsequent analyses with number of movements as the response variable.

We additionally tested for relationships between subjective HOB field assignments and all measured HOB color variables and HOB area in OLS linear models to determine if subjective color assignments aligned with our objective measurements of color.

RESULTS

Snout vent length – In the 2014–2016 data set, SVL was positively related to aggression ($F_{1,63} = 7.37$, $p = 0.009$, Fig. 1a), HOB brightness ($F_{1,91} = 7.81$, $p = 0.006$, Fig. 1b) and E2 ($F_{1,84} = 5.25$, $p = 0.024$, Fig. 1c), and negatively related to HOB saturation ($F_{1,91} = 5.98$, $p = 0.016$, Fig. 2a.) and hue ($F_{1,90} = 4.1$, $p = 0.046$, Fig. 2b).

Immunology – We found a near-significant positive regression between HOB area and bacteria killing ability ($F_{1,16} = 4.4$, $p = 0.052$, Fig. 3) but no other significant regressions between HOB color variables or sex steroids ($p > 0.1$). Five of eleven wounds induced in 2015 experienced significant tissue necrosis and only a single wound scabbed completely before the lizard entered brumation. We found no significant regression

between wound size and any HOB color predictor variable ($p > 0.2$) for the subjects that experienced necrosis and those that did not.

Sex Steroids and HOB Color – We found significant negative regressions between HOB brightness and androgens (DHT: $F_{1,81} = 5.61$, $p = 0.02$; T: $F_{1,81} = 4.82$, $p = 0.03$; Fig. 4a,b) and between HOB saturation and E2 ($F_{1,78} = 8.52$, $p = 0.005$; Fig. 5a), while the regression between HOB area and T (but not DHT) was significantly positive ($F_{1,72} = 4.19$, $p = 0.04$, Fig. 5b). We found no significant regressions between any other measures of sex steroids and color ($p > 0.1$).

Growth – In our assessment of daily growth, average days between the first and last size measurement was 29.73 (min = 5, max = 68, n = 68). The average number of measurements per male was 3.09 (min = 2, max = 8). We found a very strong negative regression between SVL growth per day and starting SVL (SVL at first measurement; $F_{1,64} = 32.28$, $p << 0.001$, Fig. 6). We found no significant regressions between measures of growth (SVL growth/day, mass gain/day, or tail growth/day) and either sex steroids or HOB color.

Aggression – In our staged aggressive encounters, we found a near-significant negative regression between aggression and E2 ($F_{1,52} = 3.09$, $p = 0.08$; Fig. 7) and a significant positive regression between aggression and HOB area ($F_{1,53} = 4.08$, $p = 0.049$; Fig. 8). We found no other significant regressions between aggression and color

or sex steroids ($p > 0.3$) and no significant regressions between aggression and any measures of growth ($p > 0.1$).

Focal observations – We detected a significant negative regression between number of hatchling male movements and temperature (Min = 20.6°C, Max = 34.4°C, mean = 26.7°C; $z_{61} = -2.49$, $p = 0.013$) and between total displays and DHT level ($z_{37} = -2.03$, $p = 0.042$). We found no other significant relationships in focal observations.

Survival – Hue was not significantly related to survival to yearling ($F_{2,128} = 0.54$, $p = 0.25$) but was significantly related to survival to adult ($F_{2,124} = 0.86$, $p = 0.024$; Fig. 9a), with individuals in the middle hue measurements surviving better. Saturation showed the opposite pattern with regard to age (survival to Yearling: $F_{2,129} = 2.23$, $p = 0.034$, survival to Adult: $F_{2,127} = 0.84$, $p = 0.11$; Fig. 9b) but, similarly to hue, those individuals in the middle measures of saturation survived best. Brightness was not significantly related to survival to adult or survival to yearling in the analysis for stabilizing selection ($p > 0.5$), but showed a consistent trend toward survival of brighter individuals (survival to Yearling: $F_{2,124} = 0.99$, $p = 0.16$; survival to Adult: $F_{2,122} = 0.76$, $p = 0.16$; Fig. 10). HOB area was not significantly related to survival to yearling ($p = 0.24$; Fig. 11) in the GLM analysis. However, the much smaller sample size available to evaluate this relationship (n=128 for spectral HOB variables; n = 86 for HOB area), paired with our observation that none of the hatchlings at the highest measures of HOB area survived

their hatchling season (Fig. 11), prompted us to investigate further. Out of 1000 replicates of 11 randomly sampled survival values from our data, 56 groups of 11 had zero survival (0.056 probability).

Pigment analysis – Three carotenoids (Beta-cryptoxanthin, Beta-carotene, and 3-Dehydrolutein) were below the level of quantification and returned a value of zero. Retention times for lutein and zeaxanthin were very close and did not form distinguishable peaks, therefore these value are reported combined. Carotenoids overall were detected at very low levels. The highest detected carotenoid (Lutein + Zeaxanthin) occurred at 26,437x lower levels than the highest detected pteridine (Drosopterin) and at 2.64x lower levels than the lowest detected pteridine (Sepiapterin; Fig. 12). We detected seven pteridine pigments and four carotenoid pigments (although Lutein + Zeaxanthin are reported combined). Raw values for carotenoid and pteridine pigments along with their color descriptions are reported in Table 1.

Subjective and objective measures of HOB – We found significant positive regressions between our field-based subjective HOB scores and two subjective orange measurements (HOB area: $F_{1,83} = 8.361$, $p = 0.005$; HOB saturation: $F_{1,90} = 4.35$, $p = 0.04$) and a near-significant positive regression with a third subjective measurement (HOB Hue: $F_{1,89} = 3.41$, $p = 0.07$). The regression of subjective HOB scores with brightness was nonsignificant ($p = 0.2$).

DISCUSSION

Integrated measures of male quality – In Chapter 1 we showed a significant positive relationship in hatchling males between HOB color and area and subsequent fitness, and hatchling female preference for males with brighter HOB. We concluded that we have strong evidence for precocial sexual selection for HOB in hatchling *Crotaphytus collaris*. Here we tested relationships between precocial sexually selected color traits and possible costs of bearing those traits on immune function, aggression, growth, and survival in hatchling male *C. collaris*.

We found that hatchling males with larger percentages of their body covered with HOB also had higher, but not quite significantly higher, bacteria killing capacity (Fig. 3), concordant with the "good genes" hypotheses prediction that "low quality" males will be incapable of fully developing ornamentation because of the cost of that development to immune function (Hamilton and Zuk 1982). The immunocompetence handicap hypothesis (ICHH; Folstad and Karter 1992) further invokes testosterone as the mediator between immune function and, not only ornamentation, but also social dominance. This hypothesis predicts that individuals with high testosterone (T) will have more developed ornaments, be socially dominant, and that increases in T will suppress immune function (Folstad and Karter 1992). Indeed, hatchling males with more T had more expansive HOB area and those with more HOB were more aggressive. However, we found no direct

link between T and aggression or immune function. Conversely, increased androgen levels were significantly negatively related to other HOB measures, as T and DHT levels increased, HOB brightness decreased (Fig. 4); and no significant correlations were detected between androgens and our three other measures of color. Further, in focal observations, males with higher DHT (but not T) moved *less* frequently.

While androgens (T, specifically) were originally hypothesized to be the sex steroid increasing ornament production and decreasing immune function, and we have found support for this, Folstad and Karter (1992) point out in their seminal paper that "any biochemical substance that is self-regulated and exerts the two-pronged effect of compromising the immune system and stimulating trait expression" is a potential mediator for their model. Furthermore, we are evaluating a precocial sexually selected signal that develops while males are still pre-reproductive (though they become reproductive soon thereafter, Chapter 1). Progesterone (P) and 17ß-estradiol (E2) are the instigating hormones of a color change similar to HOB in adult female *C. collaris* (Cooper and Ferguson 1972) and have been linked to male color development and aggression in other species (Moore et al. 1998; Weiss and Moore 2004). Thus, we investigated the influence of P and E2 on color development, immune function, aggression, and growth. We found that, like androgens, E2 is significantly negatively

correlated with a spectral HOB measure (HOB saturation; Fig. 5a) and shows a trend of negative correlation with aggression (Fig. 7), but we found no links with progesterone.

While we have shown a positive relationship between T and HOB area (Fig. 5b) we also have shown a negative relationship between androgens and brightness (Fig 4a,b), along with a negative relationship between E2 and saturation (Fig. 5a). Though the negative relationship between aggression and E2 is perhaps expected in a male, both androgens are entirely unrelated to hatchling aggression. These results are likely due to the development of this signal in pre-reproductive hatchlings, the transitory nature of HOB, and to our sampling only at peak HOB development. HOB begin to develop roughly four weeks after hatching, peak, then begin to fade near the end of the hatchling season, just before brumation (Acevedo Crosby 2015). The entire period from hatching to brumation is four months at longest and one month at shortest (in late-season hatchlings). Color development in hatchlings may not follow the established patterns of hormone-color relationships of adults. Further, male vertebrates typically experience a drop in androgen production soon after hatching/birth followed by a surge immediately prior to sexual maturity (Lombardi 1998; Norris and Lopez 2011). However, we found no evidence that either androgen increased with hatchling age (no relationship between T or DHT and SVL), but rather found that the T metabolite E2 is positively related to SVL (Fig.1). This may indicate that as individuals age they are producing more E2, or perhaps

that those who more readily convert T to E2 may not suffer the growth losses and/or produce the increased metabolic rate associated with high circulating T levels (Buchanan et al. 2001; Uller and Olsson 2003). The period of incredibly rapid development (and sexual maturity in males, Chapter 1) is likely to be hormonally complex and whichever hormone(s) induce the production of HOB are liable to surge weeks before HOB peak, at a time at which we did not sample hormones. Additionally, hormone receptor sensitivity and abundance have a powerful influence on cellular responses to circulating hormone levels. The presence and rapid fading of orange bars, or a full orange blush in both male and female neonates, points to the possibility of color provocation by maternal hormones. The organizational effects of early exposure to sex steroids is well established in oviparous animals (Hews et al. 1994; Schwabl 1996; Lipar and Ketterson 2000; Duffy et al. 2002; Lovern and Wade 2003; Warner and Shine 2008). Early organization may "prime" some individuals to be receptive or, oppositely, insensitive to circulating hormones (Meylan et al. 2012), typically by down-regulating receptors (Duffy et al. 2002). Side-blotched lizard mothers alter their E2 egg deposition based on their mate and other proximal males' color phenotypes, producing offspring with high fitness in differing environments (Lancaster et al. 2007). Furthermore, higher levels of maternal steroid deposition in developing egg yolks is linked to decreased immune function in at least one oviparous species (Rubolini et al. 2005), indicating additional potential influences on activational responses in later life.

Finally, since the ultimate measure of cost of a hatchling trait is survival, we sought to determine if survival selection gradients were detectable in any measures of HOB. Both HOB hue and saturation appear to be experiencing stabilizing selection, with individuals in the middle ranges of trait values surviving best (Fig. 9). Regressions between survival and HOB brightness and HOB area were not significant but indicate the possibility of lower survival of those with greater HOB area (Fig. 11); further randomization modeling of the data on HOB area revealed lower than expected (but not significantly lower; $p = 0.056$) survival at the highest levels of HOB area.

Different aspects of the same SSS can potentially communicate to both potential mates and same-sex rivals. HOB area and brightness are each related to indicators of male fitness (Chapter 1). Female hatchlings prefer to associate with brighter males and brighter males who do sire offspring are more likely to sire larger numbers of offspring. Female preference for HOB area has not been tested but HOB area is positively linked to a male's probability of siring offspring but not the number of offspring he sires (Chapter 1). These findings, taken together with our data (this chapter) indicate the possibility that HOB area operates as a badge, signaling dominance to rival males (Thompson and Moore 1991), while HOB brightness is a signal of quality to potential mates. Males with more HOB area have higher T levels, are more aggressive, and those with most extensive coverage survive their hatchling season at lower than expected rates despite performing

better in assays of their immunological function. This discovery is consistent with previous findings (Chapter 1), that hatchling males with greater HOB area are more likely to sire offspring. We suggest that hatchling males that allocate resources more heavily toward HOB, or are genetically or hormonally (by maternal effects) predisposed to more developed HOB, secure matings early in life then die and the remaining males invest in or are predisposed to less developed HOB and wait to mate when they are yearlings or adults. As discussed in Chapter 1, mating with smaller males could benefit females in terms of decreased risk of harm due to forced copulations and could give those females expanded choice of mates. Males with greater HOB area have higher bacteria killing capacity and are more aggressive. HOB area can, therefore, serve as an honest signal of immune function to females and of dominance to rival males during the hatchling season.

Pigment composition – Contrary to expectations of resource trade-off and immune competence hypotheses that employ carotenoids as limiting resources, males with greater HOB area (and presumably more integumental pigment deposition) performed better in bacterial killing analyses. This is, however, consistent with our finding that HOB contain few and very low levels of carotenoids. Notably, the two carotenoids capable of being converted to vitamin A (ß-carotene and ß-cryptoxanthin) and, thus serving an immune function (von Lintig 2010; Hill and Johnson 2012), were undetectable (Table 1). The rarer red ketocarotenoids (astaxanthin, canthaxanthin), which

are considered to be potentially 'expensive' to produce (Hill 1996; García-de Blas et al. 2016), were present in very low levels. However, among studies using the same method of pigment quantification (and therefore directly comparable: McLean et al. 2017; McLean et al. in review; Merkling et al. 2018; I., Medina, K.J., Rankin, A. Lutz, A. Elliott, K. Boysen, M. Melville, and D. Stuart-Fox, unpubl. data) carotenoid levels in our tissue samples were lower than those detected in any other lizard species (D. Stuart-Fox pers. comm.). This, coupled with every pteridine detected at levels occurring at least 2.6x higher levels than the carotenoid with highest returns (Table 1), clearly points to pteridines as the primary pigments producing HOB. This is consistent with findings of pteridines in other lizard species (Ortiz et al. 1963; Morrison et al. 1995; Steffen and McGraw 2007; Weiss et al. 2012; Haisten et al. 2015) and, more generally, in a variety of non-mammals (Bagnara and Matsumoto 2006).

Because pteridines are synthesized *in vivo* from apparently abundant guanosine triphosphate (Ziegler 2003) rather than obtained from the diet and bio-converted, drosopterin (the red pteridine in highest abundance in HOB) has been called a 'cheap' substitute for 'expensive' red carotenoids (McLean et al., in review). Yet, this 'cheap' signal correlates with female preference, male mating success, and offspring sex-ratio (Chapter 1), and increased bacteria killing capacity, increased aggression, and decreased survival at extreme values of hue and saturation, and at high values of HOB area (this

chapter). We report here, in agreement with findings in other lizards and previous work in this species (Chapter 1), that a pteridine-based signal is significantly correlated with measures of fitness. Thus, it is prudent to augment the few existing studies (McLean et al. 2017, McLean et al. in review) investigating the biochemical and genetic basis of pteridines. The abundance of colorless pteridines in excess of their need has been taken as an indication that the substrate (guanosine triphosphate) availability is not a limiting factor in production. However, the widespread and consistent employment of pteridine-based color indicates that these signals are evolutionarily stable. Further investigation is warranted to discover how honesty is maintained in these cases.

Concluding Remarks – In conclusion, we found that some measures of a precocial sexually selected signal (HOB area) in hatchling *C. collaris* positively relate to immune function, aggression, and T, while other measures of the same signal (brightness) relate negatively with sex-steroid levels. Further, stabilizing selection is in evidence on HOB hue and saturation, while a trend towards higher and lower survival was evident in individuals with high brightness and more HOB area, respectively. We suggest that these findings taken together with those of Chapter 1 point to a dual signaling system in this species in which hatchling males either 1) produce more developed HOB, mate early, then die (i.e., precocial sexual selection), or 2) produce less developed HOB and wait to mate as yearlings and adults, subsequent to the development of characteristically blue-

green-yellow adult coloration (i.e., classical sexual selection). Further, we add here to the mounting evidence that pteridine pigmentation is a critical contributor to red/orange signals in non-avian reptiles and call for further exploration into its selective maintenance.

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Table 1. Pigments isolated from two HOB skin samples from one *C. collaris* hatchling. Response is the raw mean of the response/sample weight of the two samples tested combined. A response of zero indicates the pigment was below the level of detection.

	Pigment	Response	Color Produced
Pteridine	Drosopterin	1375766.61	Red
	Isoxanthopterin	205593.11	Purplish/UV
	Pterine-6-COOH	14403.42	Colorless/UV
	Pterin	7687.50	Colorless/UV
	6-Biopterin	6332.05	Colorless/UV
	Xanthopterin	402.80	Yellow
	Sepiapterin	137.65	Yellow
Xanthophyll Carotenoid	Lutein+Zeaxanthin	52.04	Yellow, Orange-Red
	Canthaxanthin	0.47	Red
	Astaxanthin	0.42	Red
	Beta-cryptoxanthin	0	Yellow
	3-Dehydrolutein	0	Yellow
Carotene Carotenoid	Beta-carotene	0	Orange-Red

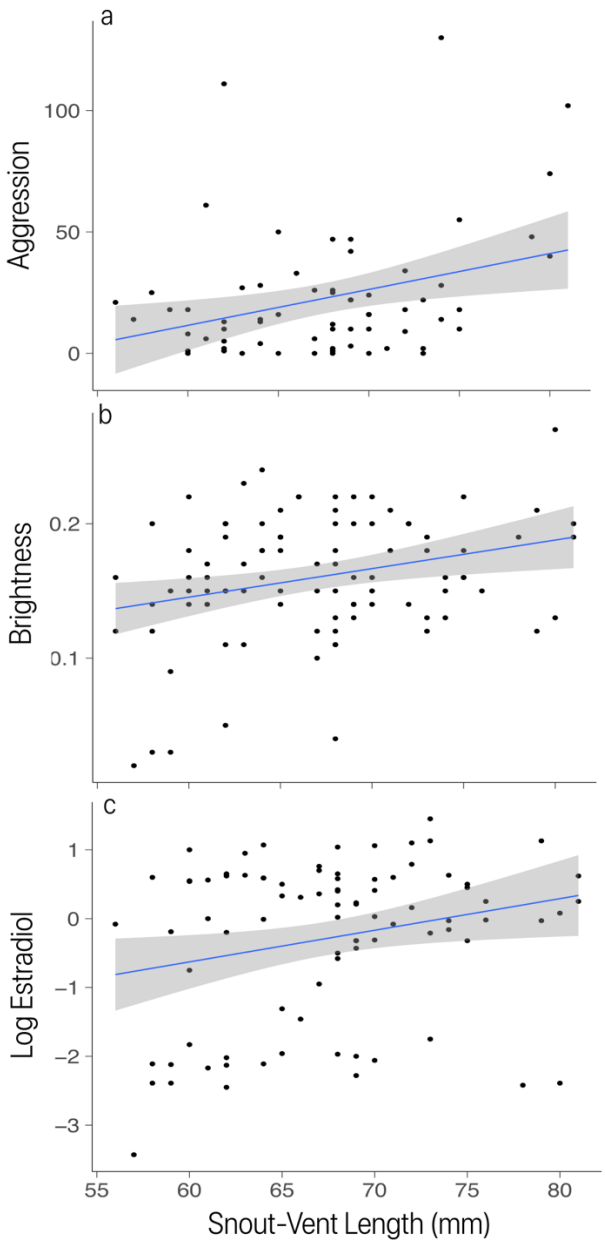

Fig. 1. Significant positive regressions between a) Aggression ($F_{1,63}$ = 7.37, p = 0.009), b) HOB Brightness ($F_{1,91}$ = 7.81, p = 0.006), and c) Log Estradiol ($F_{1,84}$ = 5.25, p = 0.024), and Snout-Vent Length. Grey shading represents the 95% confidence interval.

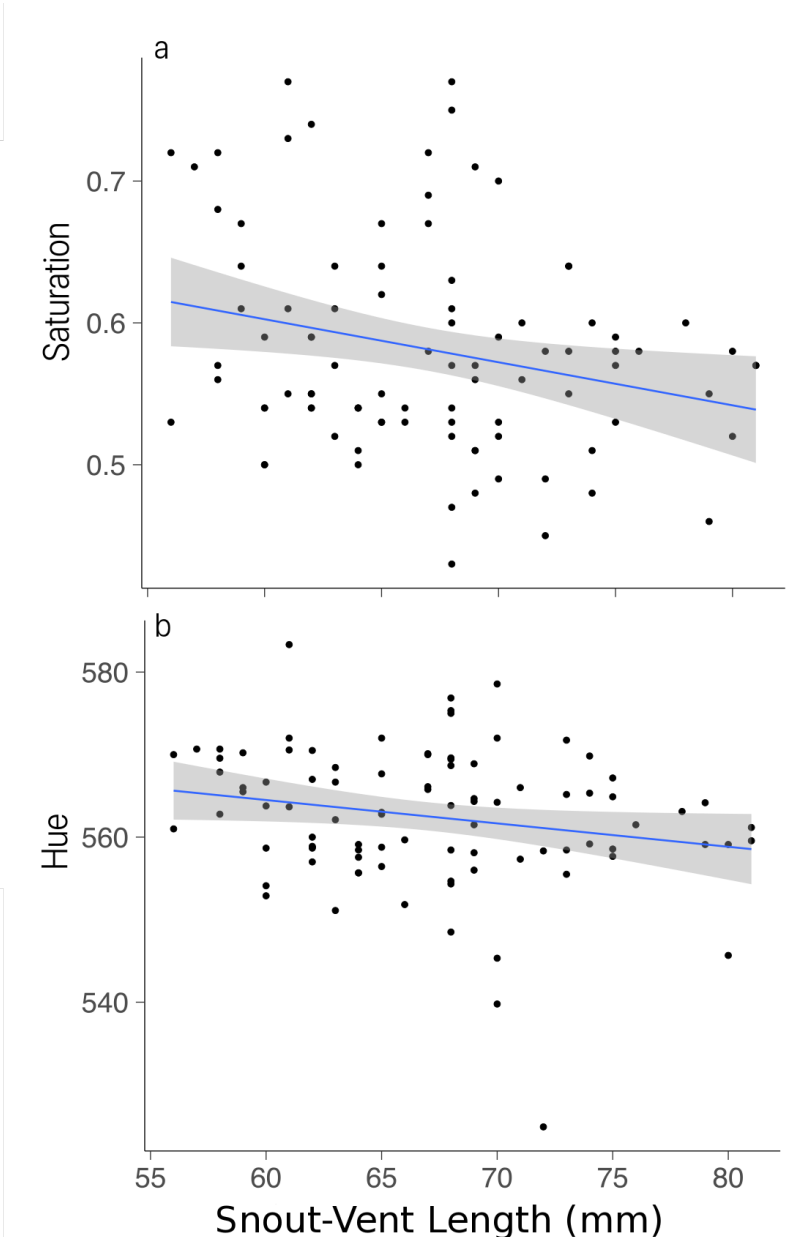

Fig. 2. Significant negative regressions between a) HOB Saturation ($F_{1,91} = 5.98$, $p = 0.016$), b) HOB Hue ($F_{1,90} = 4.1$, $p = 0.046$) and Snout-Vent Length. Grey shading represents the 95% confidence interval.

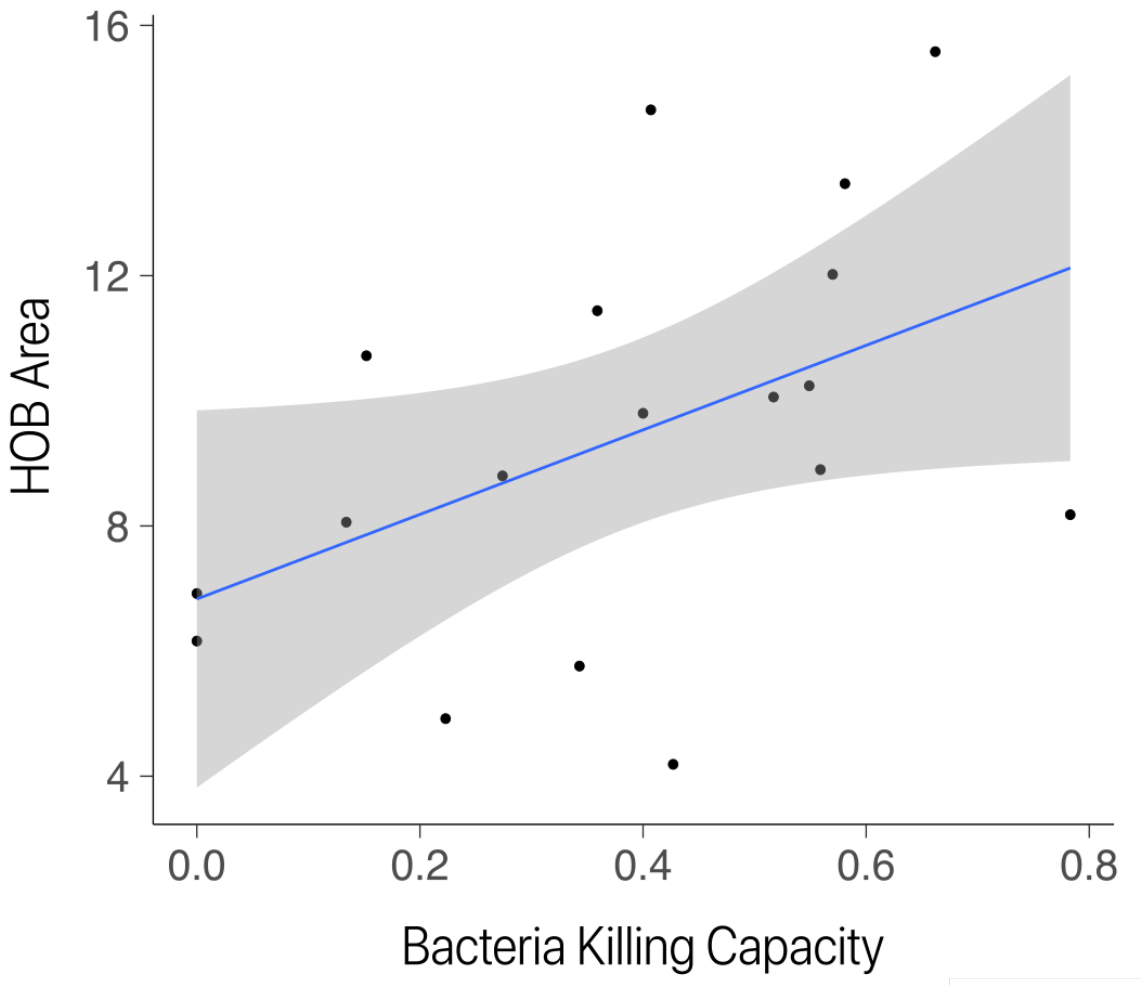

Fig. 3. Near significant positive regression between HOB area and bacteria killing capacity ($F_{1,16} = 4.4$, $p = 0.052$). Grey shading represents the 95% confidence interval.

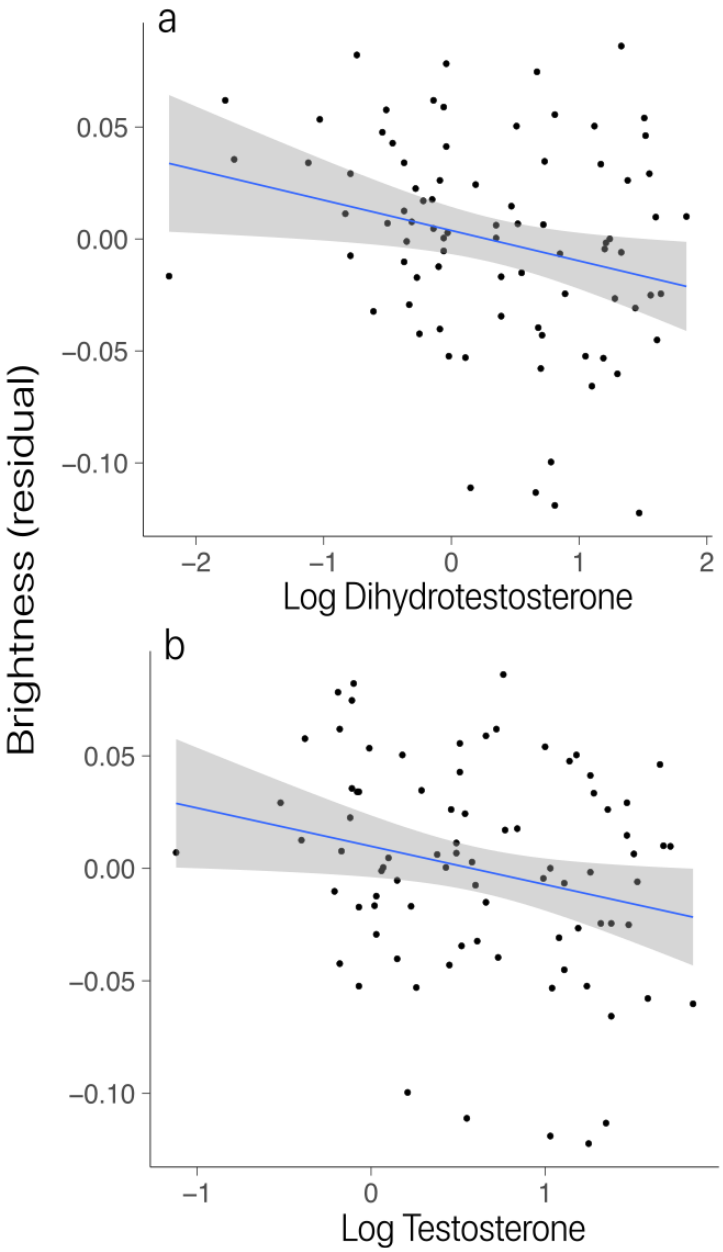

Fig. 4. Significant negative regressions between HOB brightness and a) dihydrotestosterone ($F_{1,81}$ = 5.61, p = 0.02) and b) testosterone ($F_{1,81}$ = 4.82, p = 0.03). Dihydrotestosterone and testosterone are log transformed. Brightness measures are residuals of the regression with SVL. Grey shading represents the 95% confidence interval.

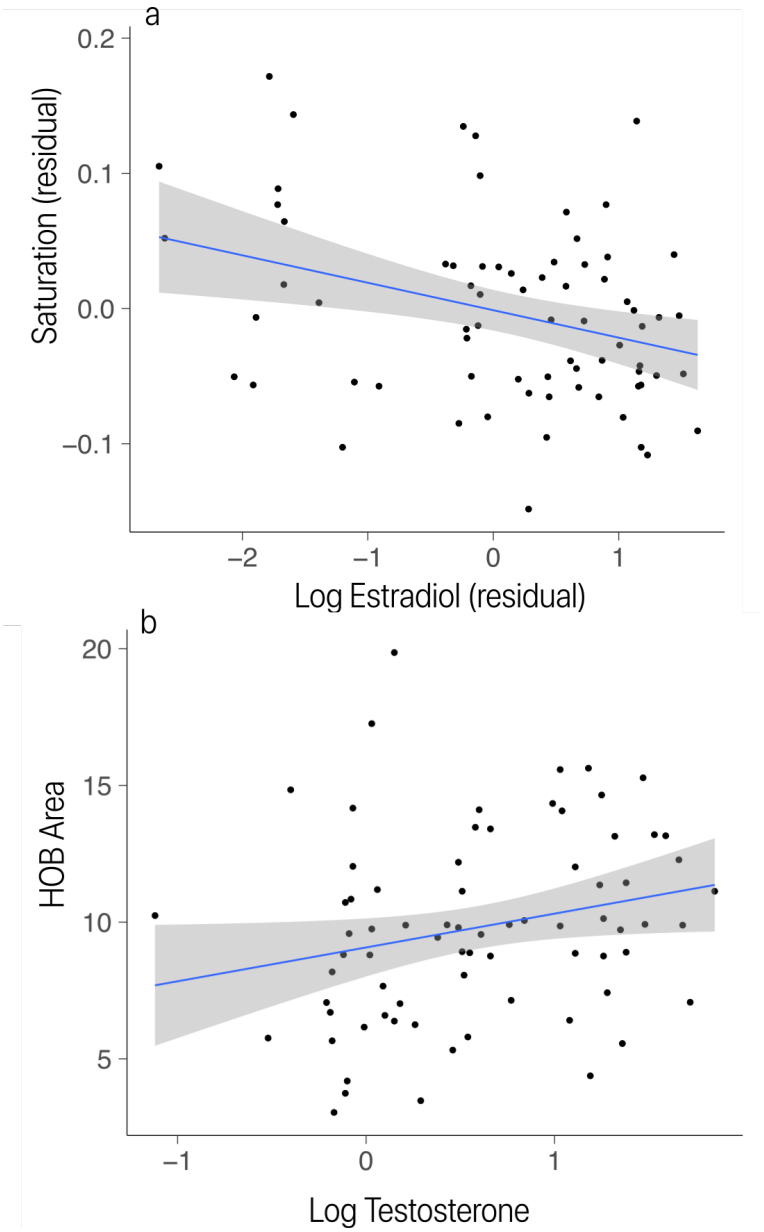

Fig. 5. Significant regressions between a, HOB Saturation and Log Estradiol ($F_{1,78}$= 8.52, p = 0.005) and b, HOB Area and Log Testosterone ($F_{1,72}$ = 4.19, p = 0.04). Testosterone and Estradiol are log transformed and Log Estradiol and Saturation are residuals of the regression with SVL. Grey shading represents the 95% confidence interval.

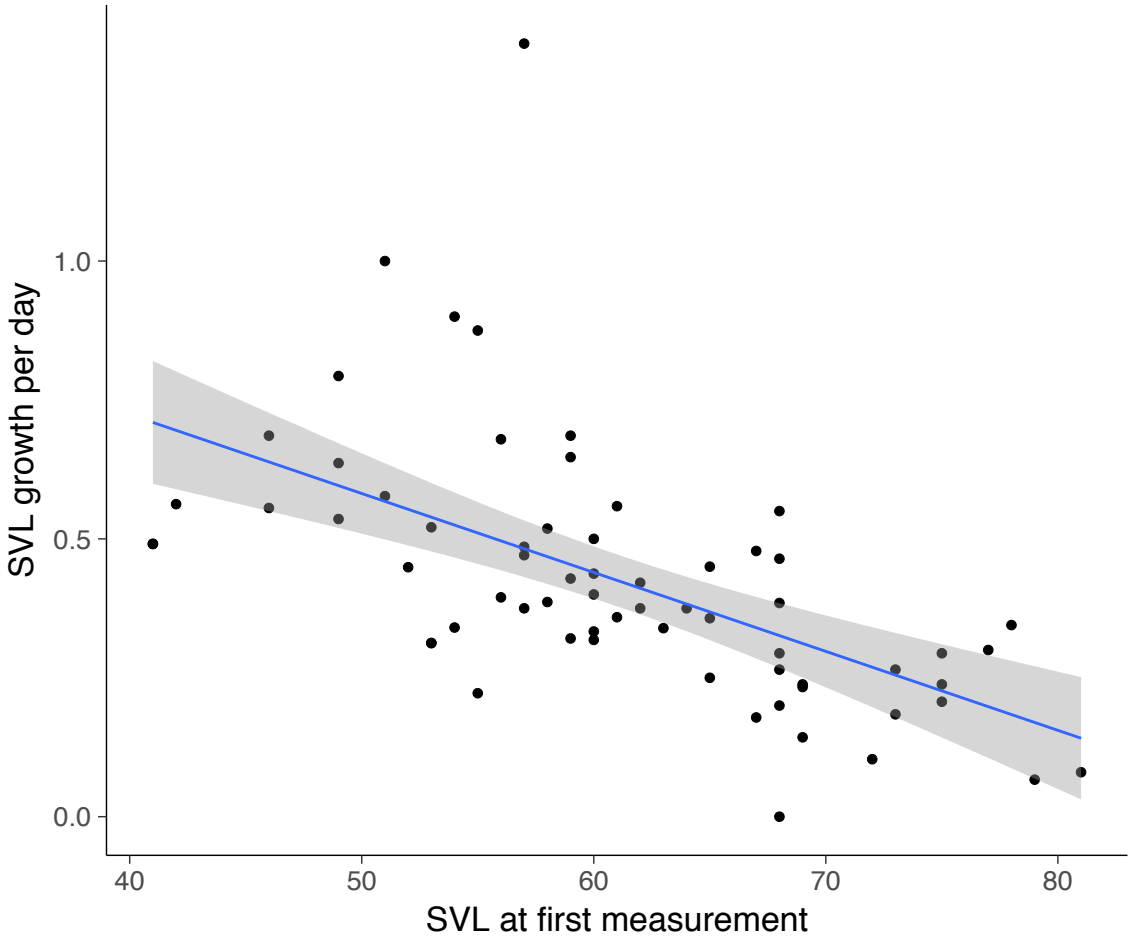

Fig. 6. Significant negative regression between SVL growth per day and SVL at first measurement ($F_{1,64} = 32.28$, $p << 0.001$). Grey shading represents the 95% confidence interval.

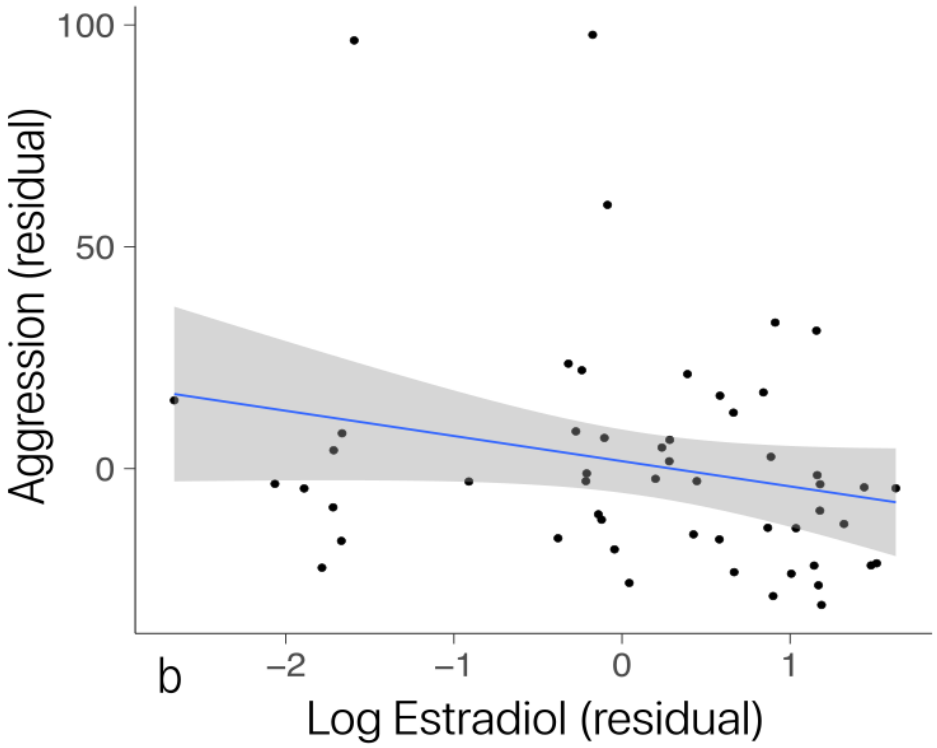

Fig. 7. Near significant negative regression between Aggression and Log Estradiol ($F_{1,52} = 3.09$, $p = 0.08$). Aggression and Log Estradiol are both residuals of the regression with SVL. Grey shading represents the 95% confidence interval.

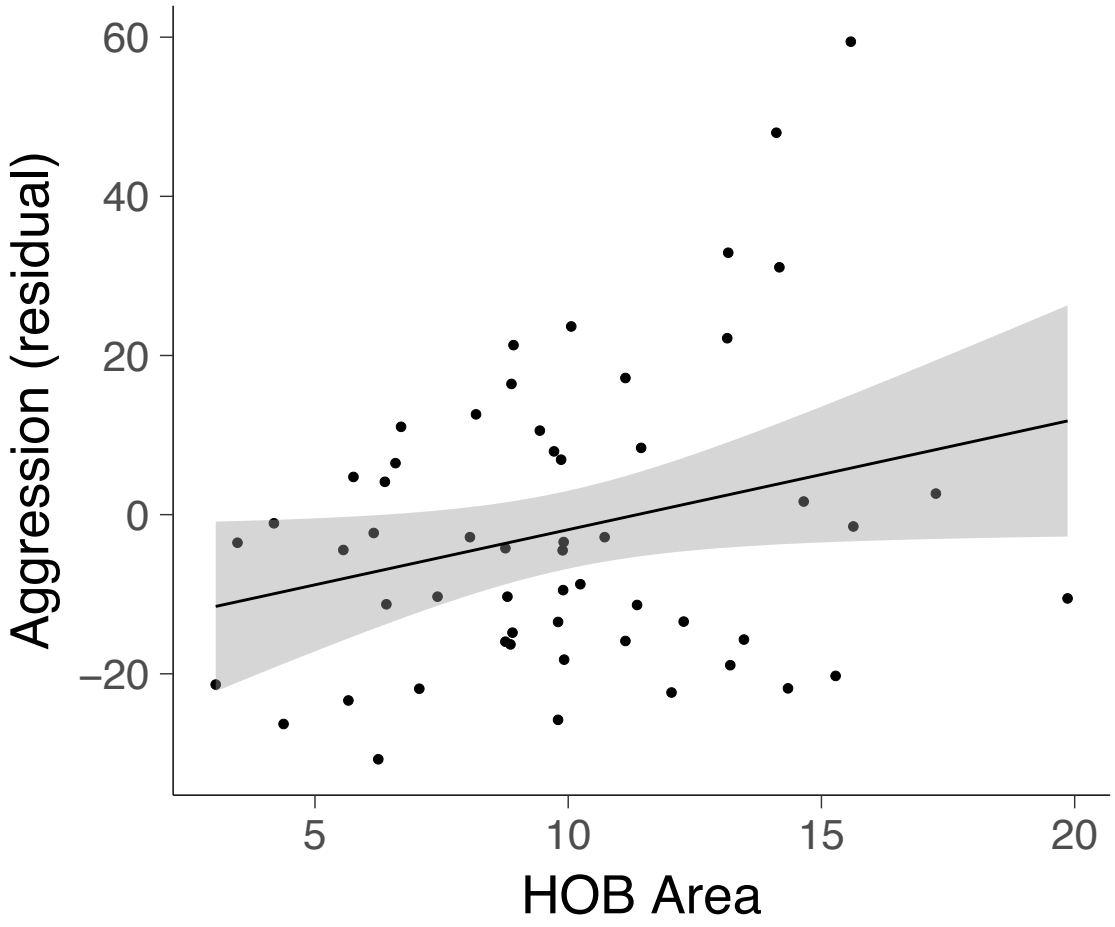

Fig. 8. Significant positive regression between Aggression and HOB Area ($F_{1,53} = 4.08$, $p = 0.049$). Aggression is the residuals of the regression with SVL. Grey shading represents the 95% confidence interval.

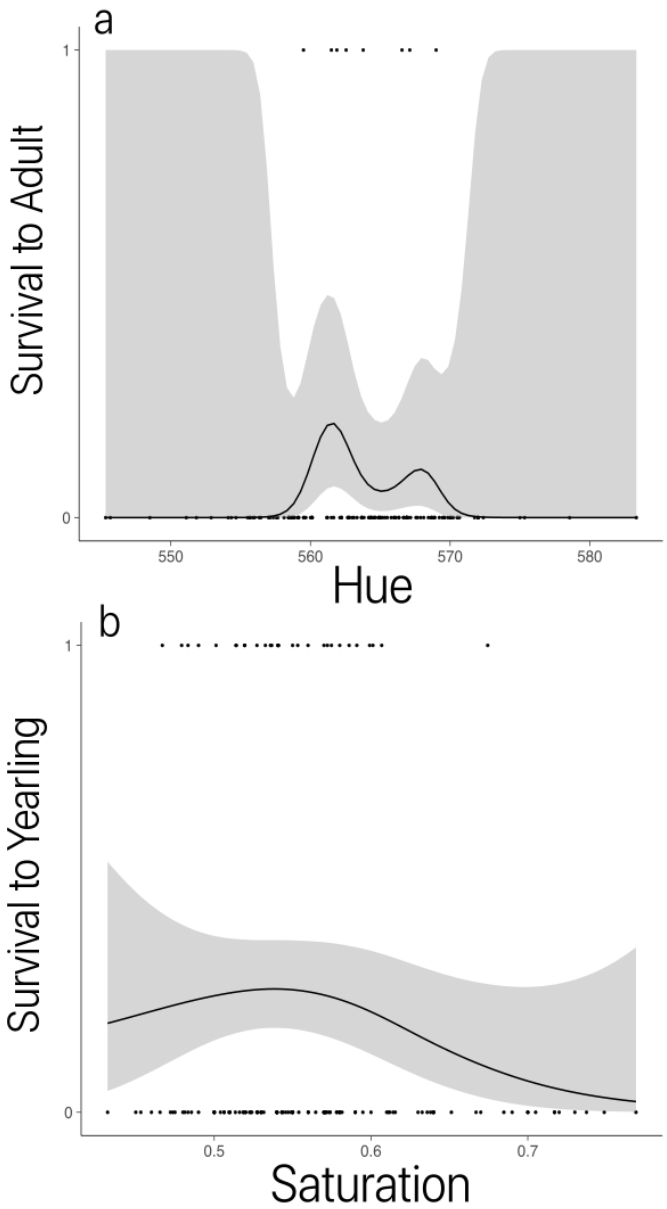

Fig. 9. Significant regressions between a, survival to adult and hue ($F_{2,124}$ = 0.86, p = 0.024) and b, survival to yearling and saturation ($F_{2,129}$ = 2.23, p = 0.034). Grey shading represents the 95% confidence interval.

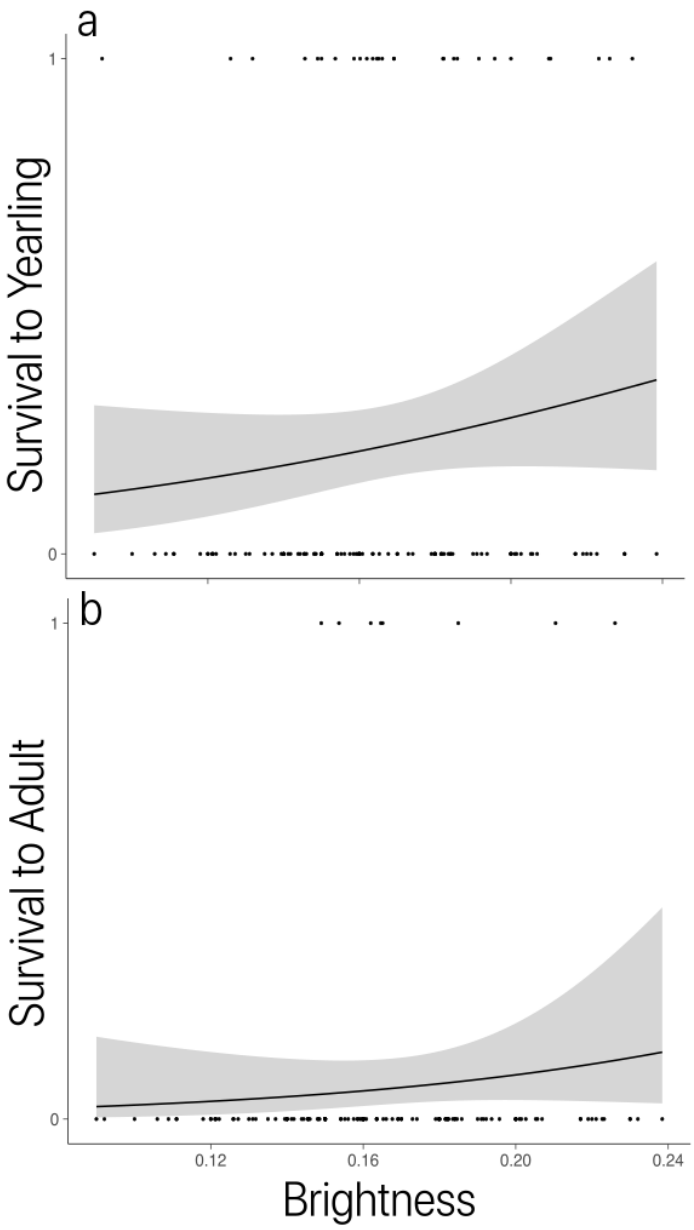

Fig. 10. Regressions between a, brightness and survival to yearling ($F_{2,124} = 0.99$, $p = 0.16$) and b, brightness and survival to adult ($F_{2,122} = 0.76$, $p = 0.16$). Grey shading represents the 95% confidence interval.

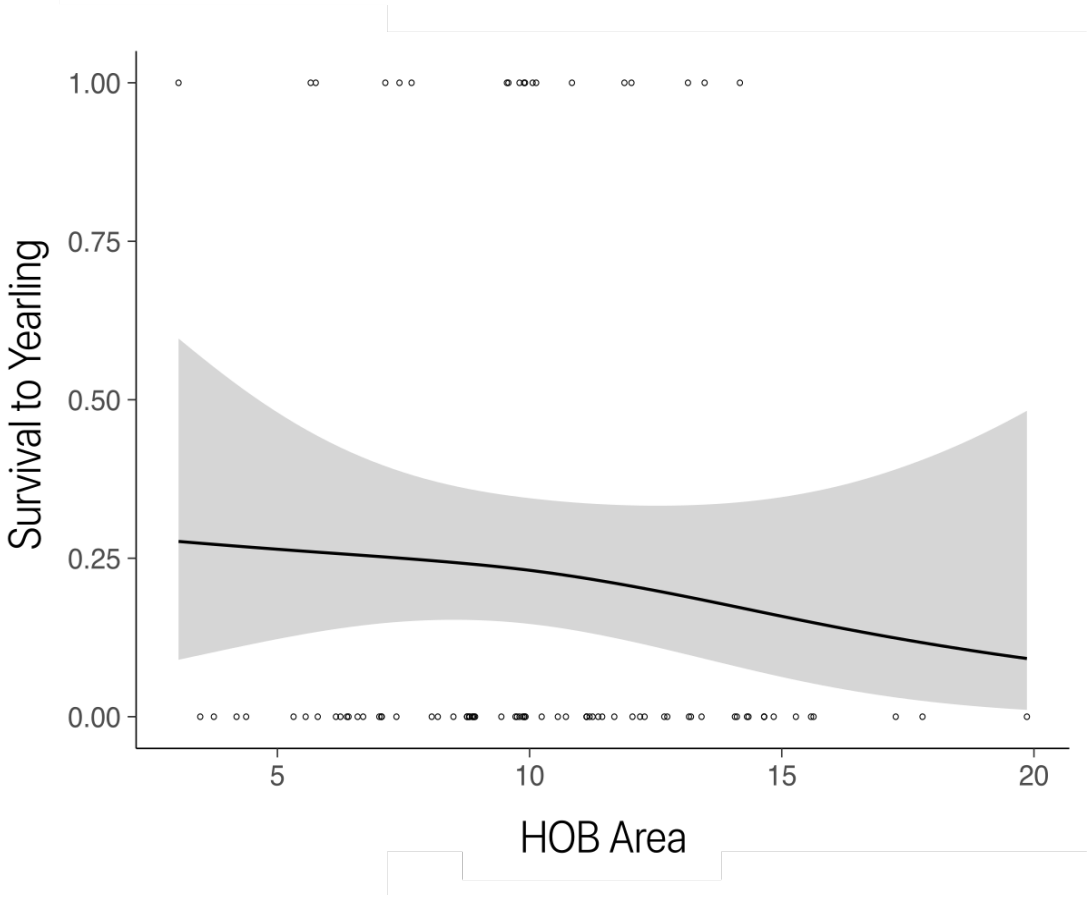

Fig. 11. Non-significant regression between survival to yearling and HOB area ($F_{2,83}$ = 0.78, p = 0.24) showing the decline in survival at highest measures of HOB area. Grey shading represents the 95% confidence interval.

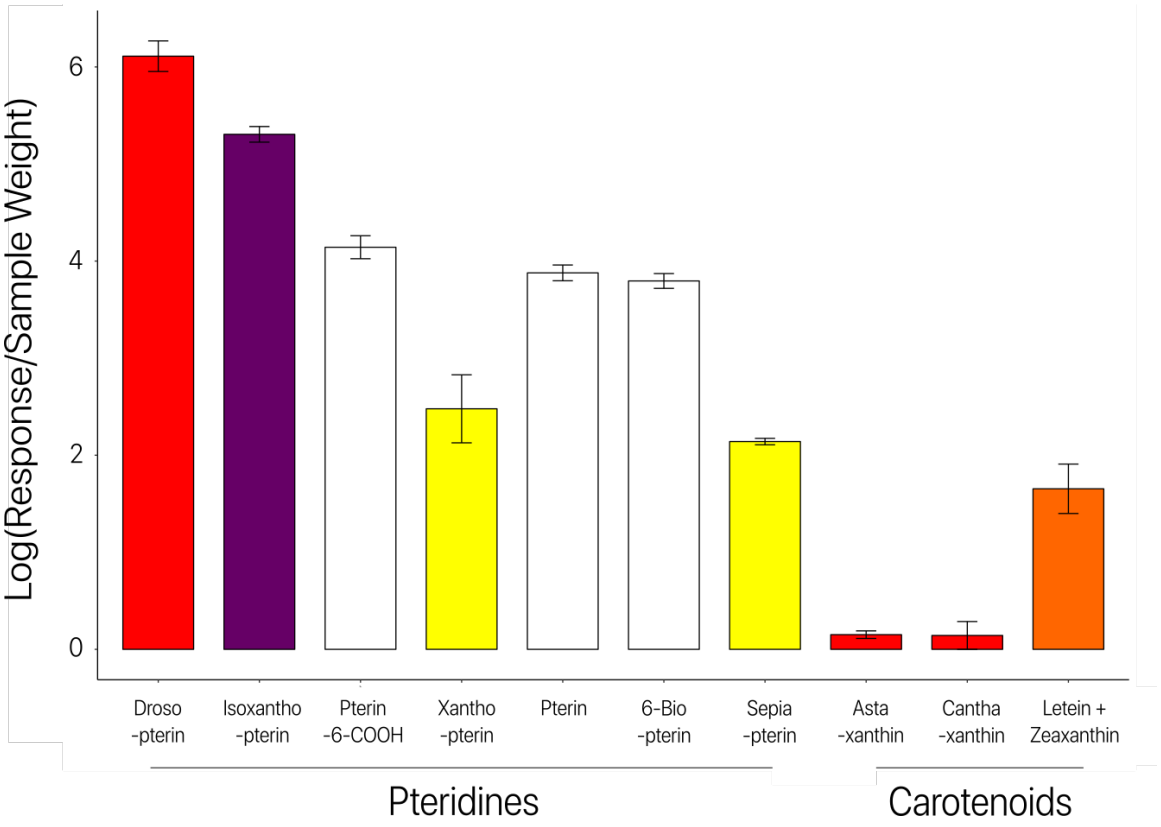

Fig. 12. Mean ± SE pteridine and carotenoid levels detected in two skin samples of *C. collaris* HOB. Raw values are + 1 log transformed for visualization. Bar colors represent the color produced by the pigment.

VITA

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Gene dosage in the eastern collared lizard (*Crotaphytus collaris)*
Identifying prey of the Ozark Big Eared Bat (*Corynorhinus townsendii)*

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Co-Coordinator – Diversity Luncheon for the Evolution Conference
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